# The regulatory mechanisms of enhancing IGF-1R expression and activity and the impact on therapy resistance of melanoma cells

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## Erklärung:

Ich erkläre hiermit, dass ich die zur Promotion eingereichte Arbeit selbstständig verfasst und nur die angegebenen Quellen und Hilfsmittel benutzt habe.

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The data in this dissertation have been published.

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J.Wang: designed, performed and analyzed most of the experiments and wrote the manuscript;

T.Sinnberg and H.Niessner: performed stability assay;

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F.Meier: discussed the results and commented on the manuscript;

N. Leslie: provided sequence of PTENs;

B.Schittek: conceived and supervised the project and wrote the manuscript.

## Abbreviations:

| | |
|---|---|
| $Amp_r$ | Ampicillin resistance gene |
| AKT3 | V-Akt Murine Thymoma Viral Oncogene Homolog 3 |
| ATCC | American Type Culture Collection |
| APC | Anaphase Promoting Complex |
| AURK | Aurora Kinase |
| BCL2 | B-Cell Lymphoma 2 |
| BCL-XL | B-Cell Lymphoma extra large |
| bp | Base pair |
| BRAF | Murine Sarcoma Viral (V-Raf) Oncogene Homolog B1 |
| BSA | Bovine Serum Albumin |
| cDNA | Complementary DNA |
| CHX | Cycloheximide |
| c-KIT | V-Kit Hardy-Zuckerman 4 Feline Sarcoma Viral Oncogene Homolog |
| CMV | Cytomegalovirus |
| c-Myc | V-Myc Avian Myelocytomatosis Viral Oncogene Homolog |
| Crk | V-Crk Avian Sarcoma Virus CT10 Oncogene Homolog |
| CTLA4 | Cytotoxic T-lymphocyte antigen 4 |
| C3G | Rap Guanine Nucleotide Exchange Factor (GEF) 1 |
| DMEM | Dulbecco's Modified Eagle's Medium |
| DMSO | Dimethyl sulfoxide |
| DNA | Deoxyribonucleic acid |
| dsDNA | Double-stranded DNA |
| DSB | DNA double-strand break |
| DTIC | Dacarbazine |
| EGF | Epidermal Growth Factor |
| EMEA | European Agency for the Evaluation of Medicinal Products |
| ERK | Mitogen-Activated Protein Kinase |
| ER | Endoplasmic Reticulum |
| EDTA | Ethylene Diamine Tetraacetic Acid |
| FAK | Focal Adhesion Kinase |
| FBS | Fetal Calf Serum |
| FDA | Food and Drug Administration |
| FF | Foreskin Fibroblast |
| GAPDH | Glyceraldehyde-3-Phosphate Dehydrogenase |
| GFP | Green fluorescent protein |
| Grb10 | Growth Factor Receptor-Bound Protein 10 |
| Grb2 | Growth Factor Receptor-Bound Protein |
| HAUSP/ USP7 | Herpes virus-associated ubiquitin-specific protease |
| HA | Hemagglutinin |
| HIV-1 | Human Immunodeficiency Virus 1 |
| HRP | Horseradish Peroxidase |
| $IC_{50}$ | Half maximal inhibitory concentration |
| IGF | Insulin-like Growth Factor |
| IGF-1 | Insulin-like Growth Factor 1 |

| | |
|---|---|
| IGFBP | IGF Binding Proteins |
| IGF-1R | Insulin-like Growth Factor 1 Receptor |
| IGF-2R | Insulin-like Growth Factor 2 Receptor |
| IR | Insulin Receptor |
| IRS-1/2 | Insulin-Receptor Substrate1/2 |
| kDa | Kilo dalton |
| kb | Kilo base |
| LB medium | Lysogeny Broth medium |
| MAPK | Mitogen-Activated Protein Kinase |
| MEK1/2 | Mitogen-Activated Protein Kinase Kinase 1/2 |
| miR-30 | MicroRNA-30 |
| ml | Millilitre |
| mRNA | Messenger RNA |
| mTOR | Mechanistic Target Of Rapamycin (Serine/Threonine Kinase) |
| MUH | 4-Methylumbelliferylheptanoate |
| M6P | Mannose-6-Phosphate |
| NEDD4 | Neural Precursor Cell Expressed, Developmentally Down-Regulated 4, E3 Ubiquitin Protein Ligase |
| NEJM | New England Journal of Medicine |
| NRAS | Neuroblastoma RAS Viral (V-Ras) Oncogene Homolog |
| dNTP | Desoxyribonucleosid-5´-triphosphate |
| nt | Nucleotides |
| PBS | Phosphate Buffered Saline |
| PDK | Phosphoinositide-Dependent Kinases |
| PDK1/2 | Phosphoinositide-Dependent Kinases 1/2 |
| PD-1 | Programmed Death protein-1 |
| PD-L1 | Programmed Death-Ligand 1 |
| PFS | Progression Free Survival |
| PFA | Para-formaldehyde |
| PGK | Phosphoglycerate Kinase |
| PI | Propidium Iodide |
| PIP2 | Phosphatidylinositol-4,5-bisphosphate |
| PIP3 | Phosphatidyl Inositol-3,4,5-triphosphate |
| PI3K | Phosphatidyl Inositol-3 Kinase |
| PKC | Protein Kinase C |
| PLK1 | Polo-Like Kinase 1 |
| PPP | Picropodophyllin |
| PPGK | Murine Phosphoglycerate Kinase (PGK) promoter |
| PSA | Prostate-Specific Antigen |
| PTEN | Phosphate and Tensin homologue |
| PTPs | Tensin/auxilin and protein Tyrosine Phosphatases |
| $Puro_r$ | Puromycin resistance gene |
| PVDF | Polyvinylidene difluoride |
| qRT-PCR | Quantitative Real-Time PCR |
| Rap1 | Ras-related protein 1 |
| RAS | Rat sarcoma |
| RGP | Radial Growth Phase |
| RNA | Ribonucleic Acid |
| rpm | Revolutions Per Minute |

| | |
|---|---|
| RPMI | Roswell Park Memorial Institute |
| RT | Room Temperature |
| RTK | Receptor Tyrosine Kinases |
| shRNA | Short hairpin RNA |
| SHC | Src Homology/Collagen |
| siRNA | Small interfering RNA |
| SOCS | Suppressors Of Cytokine Signaling |
| SOS | Son Of Sevenless |
| STATs | Signal Transducers and Activators of Transcription |
| TBE | Tris Borate EDTA |
| TBST | Tris Buffered Saline with Tween20 |
| TE | Tris-EDTA |
| TET | Tetracycline |
| TETO | tet operator |
| TEP1 | TGF-β regulated and Epithelial cell enriched Phosphatase |
| TRAIL | Tumor-necrosis-factor-Related Apoptosis-Inducing Ligand |
| VGP | Vertical-Growth Phase |
| VSV-G | Vesicular Stomatitis indiana Virus protein G |

# 1 Introduction

Melanoma is a malignant tumor, which originates from benign melanocytes (Slominski, Tobin et al. 2004; Gray-Schopfer, Wellbrock et al. 2007). Due to the production of melanin, the majority of melanomas are black or brown. It predominantly occurs in skin, but is also found in some other body locations as in the bowel and in the eye. There are four basic types of melanoma, which differ in frequency and location in the body. Superficial spreading melanoma, nodular melanoma, and lentigo maligna melanomas make up 90% of all diagnosed malignant melanomas. Acral lentiginous melanoma and a few very rare types together make up the other 10% (Clark 1967; Reed 1976). Melanoma is the least common but the most deadly skin cancer. Although it´s accounting for only about 4% of all cases, it results in 80% of skin cancer deaths due to its potential to metastasize early in tumor development (Miller and Mihm 2006; Gray-Schopfer, Wellbrock et al. 2007).

## 1.1 Incidence and risk factors

At the present time, the incidence of melanoma is doubling every 10 to 20 years (Sandru, Voinea et al. 2014). For young people aged between 25 and 49 the mortality rate increases in the magnitude of 2-3% per year in some North and West European countries and up to 8% in Spain. For old people the mortality increase varied in men above age 70 between 2.7% (Netherlands) and 7.5% (Spain) and in elderly women between 0.8% (Norway) and 7.7% (Spain) (de Vries, Bray et al. 2003).

Risk factors for melanoma development can be subdivided into genetic and environmental with interaction between the two. About 5% of all invasive cutaneous melanomas run in a familial setting with two or more close relatives affected. This observation indicates that low prevalence/high penetrance genes are responsible for a small minority of melanoma patients (MacKie, Hauschild et al. 2009). In addition, the incidence of melanoma increases at higher rates in Australia and North America. This geographic pattern reflects that ultraviolet light exposure is one of the primary causes of melanoma development. By this, people with white skin are more susceptible to UV-induced DNA damage (*World Cancer Report 2014*). Moreover, high counts of benign melanocytic naevi, the effect of female sex hormones,

socioeconomic status, occupation, exposure to pesticides and ingestion of therapeutic drugs including immunosuppressive and non-steroidal anti-inflammatory drugs are considered to be risk factors for melanoma development (MacKie, Hauschild et al. 2009).

## 1.2 Prognosis and therapy

### 1.2.1 Prognosis

Factors that affect prognosis are tumor thickness in millimeters (Breslow's depth), depth related to skin structures (Clark level), type of melanoma, presence of ulceration, presence of lymphatic/perineural invasion, presence of tumor-infiltrating lymphocytes (if present, prognosis is better), location of lesion, presence of satellite lesions, and presence of regional or distant metastasis (Homsi, Kashani-Sabet et al. 2005). Thick melanomas are associated with a worse prognosis. It´s generally considered that the cancer is incurable when distant metastases occur, especially metastases in brain, bone and liver, and the five-year survival rate is less than 10% (Balch, Buzaid et al. 2001).

### 1.2.2 Therapy

#### 1.2.2.1 Surgical treatment

The majority of melanoma patients have early stage disease, with 62.6% presenting with stage 0 or I disease, and an additional 23.1% with stage II disease (Chang, Karnell et al. 1998). The standard therapy for localized melanoma is surgical resection, which can achieve a high cure rate. Moreover, recent studies showed that advanced-stage melanoma patients get a benefit from surgical excision (Young, Martinez et al. 2006). In a phase 2 trial, complete resection for stage IV melanoma prolonged overall survival with a median survival of 21 months (Sosman, Moon et al. 2011).

#### 1.2.2.2 Chemotherapy

Chemotherapy has been the standard systemic treatment for patients with disseminated melanoma for many years. There are few cytotoxic agents that have been approved for the treatment of malignant melanoma. As one of the only Food and Drug Administration (FDA)-approved cytotoxic agents for the treatment of metastatic melanoma, Dacarbazine

(DTIC) has limited benefit in the adjuvant treatment of melanoma (Eigentler, Caroli et al. 2003; Bedikian, DeConti et al. 2011; Chapman, Hauschild et al. 2011). Although combination chemotherapy regimens with or without cytokines (biochemotherapy) or tamoxifen increased response rates, it has not been demonstrated to be superior to monotherapy with DTIC in terms of survival (Eggermont and Schadendorf 2009). Carmustine, paclitaxel (Taxol), temozolomide and cisplatin, among others have been tested in limited clinical studies, but all of these therapies provide little benefit to overall patient survival (Tarhini and Agarwala 2006; Gray-Schopfer, Wellbrock et al. 2007).

#### 1.2.2.3 Immunotherapy

In parallel with the development of BRAF inhibitors there are advances in immunotherapy. The cytotoxic T-lymphocyte antigen 4 (CTLA-4) expressed on the surface of activated T cells limits the development of antigen specific immune responses and prevents uncontrollable overactivation of immune effector mechanisms (Melero, Hervas-Stubbs et al. 2007). Ipilimumab is an anti–cytotoxic CTLA-4 antibody, which acts to upregulate antitumor immunity. Two phase III randomized clinical trials demonstrate that it significantly improved median overall survival (Hodi, O'Day et al. 2010; Robert, Thomas et al. 2011). In long-term follow up of earlier studies three separate trials achieved 5-year survival rates of 13, 23 and 25%, respectively (Prieto, Yang et al. 2012).

The programmed death protein-1 (PD-1) is an inhibitory co-receptor, which is expressed on activated T cells or B cells. Its ligand B7-H1, programmed death-ligand 1 (PD-L1), mediates PD-1-dependent immunosuppression. The interaction of PD-L1 expressed on cancer cells or tumor stromal cells with PD-1 on the surface of T cells facilitates tumor escape from antitumor immunity (Blank and Mackensen 2007; Jin, Ahmed et al. 2011). FDA has approved two anti-PD-1 antibodies, pembrolizumab and nivolumab, targeting the PD-1 receptors for the treatment of metastatic melanoma in 2014. Compared with chemotherapy, superior efficacy of nivolumab has been demonstrated in previously untreated patients with wild-type BRAF tumors (Robert, Long et al. 2015). Similar effects are seen in patients with either mutant or wild-type BRAF tumors after progression during ipilimumab therapy and in patients with

tumors positive for BRAF mutation after progression during treatment with a BRAF inhibitor (Weber, D'Angelo et al. 2015).

Ipilimumab is considered to be able to prolong overall survival, while nivolumab produces durable tumor regression in patients with melanoma. Based on their distinct immunologic mechanisms of action and supportive preclinical data, trials of the combination of nivolumab with ipilimumab in patients with advanced melanoma were conducted. Recently a randomized, double blind, phase 3 study showed that among previously untreated patients with metastatic melanoma progression free survival (PFS) was significantly prolonged by treatment with nivolumab alone or combined with ipilimumab than ipilimumab alone. In patients with PD-L1–negative tumors, nivolumab combined with ipilimumab was more effective than either agent alone (Larkin, Hodi et al. 2015).

#### 1.2.2.4 Targeted therapy with signal transduction inhibitors

In melanoma there are three well-defined driver oncogenes: BRAF, NRAS and c-KIT, which activate predominantly the MAPK signaling pathway (Swick and Maize 2012).

##### 1.2.2.4.1 BRAF inhibitor

Somatic mutations in BRAF exist in 40-50% of metastatic melanoma (Maldonado, Fridlyand et al. 2003; Curtin, Fridlyand et al. 2005). In the vast majority of mutations the valine at the 600th amino acid position is substituted by glutamine in 80% of cases (V600E) (Rubinstein, Sznol et al. 2010; Lovly, Dahlman et al. 2012). PLX4032 (vemurafenib) specifically targets the $BRAF^{V600}$ mutation and in a multi-center phase II trial of patients with this mutation PLX4032 resulted in significantly longer median overall survival than chemotherapeutic agents (Sosman, Kim et al. 2012). PLX4032 was approved by the FDA in 2011 and by European Agency for the Evaluation of Medicinal Products (EMEA) in 2012 for first-line treatment of patients with $BRAF^{V600}$-mutation positive melanoma. Although the evidence for clinical benefit with PLX4032 is compelling, resistance has posed a major obstacle (Johannessen, Boehm et al. 2010; Nazarian, Shi et al. 2010; Villanueva, Vultur et al. 2010). Additionally, an increased incidence of cutaneous squamous cell carcinoma has been associated with PLX4032 treatment. The paradoxical MAPK pathway activation rather than

PLX4032 has been identified as the culprit (Su, Viros et al. 2012). Dabrafenib (GSK2118436, GlaxoSmithKline) is another specific $BRAF^{V600}$ inhibitor, which has been found to have preclinical activity against melanoma in cell lines and xenografts. Early phase trials also demonstrated that it has superior efficacy against BRAF mutant melanoma (Falchook, Long et al. 2012). Objective responses to it were seen in 53-69% of patients with $BRAF^{V600E}$ or $^{V600K}$ mutations. Its similarity to PLX4032 is timing of responses and development of resistance, but its side-effect profile is different from PLX4032 in several ways. Compared with PLX4032, it appeared to result in a lower incidence of cutaneous squamous cell carcinomas or keratoacanthomas (6-19%) (Johnson and Sosman 2013).

#### 1.2.2.4.2 MEK inhibitor

The blockade of MEK in the MAPK pathway downstream from BRAF mechanistically could have activity in both, melanoma with BRAF mutations and NRAS mutations. Trametinib is a highly specific inhibitor of MEK1/ 2 and best evaluated in the clinic (Infante, Fecher et al. 2012). A phase III trial demonstrated that in 322 patients with melanoma positive for $BRAF^{V600E}$ or $^{V600K}$ mutations, the median duration of PFS was 4.8 and 1.5 months, the 6-month overall survival rate was 81 and 67%, and objective response rates were 22 and 8% for trametinib and chemotherapy, respectively (Flaherty, Robert et al. 2012).

#### 1.2.2.4.3 Combination of Dabrafenib and Trametinib

It´s postulated that bypass activation of the MAPK pathway was considered to be an important mechanism of drug resistance, hence complete abrogation of the MAPK pathway by simultaneous administration of BRAF and MEK-targeting agents was hoped to prevent or delay the development of acquired resistance to BRAF inhibitors. However recent publications showed that the emergence of resistance was delayed only 0.5 to 3.7 months by such combinations in terms of PFS in the New England Journal of Medicine (NEJM) reports (Larkin, Ascierto et al. 2014; Long, Stroyakovskiy et al. 2014; Robert, Long et al. 2015). The basic implications of these outcomes have been revisited, and the underlying biology is needed to be investigated in future.

#### 1.2.2.4.4 c-KIT inhibitor

Somatic mutations in KIT, the V-Kit Hardy-Zuckerman 4 Feline Sarcoma Viral Oncogene Homolog, are more often found in primary melanoma tumors on acral surfaces (soles of the feet, nail beds) or in mucosae (10-20%) (Satzger, Schaefer et al. 2008; Torres-Cabala, Wang et al. 2009). Imatinib is a kinase inhibitor, which inhibits a range of tyrosine kinases including c-KIT and is currently FDA approved for gastrointestinal stromal tumors (Ugurel, Hildenbrand et al. 2005). Two phase II trials have been completed using imatinib in KIT mutant melanoma. The data indicates an objective response rate of 16% and 23%, and a median PFS of 3 and 3.5 months, respectively, for the two separate trials (Carvajal, Antonescu et al. 2011).

## 1.3 Biology of Melanoma

### 1.3.1 Biologic Events and Molecular Changes in the Progression of Melanoma

It is believed that the development of melanoma is involved in a multi-step process, due to complex interactions between genetic and environmental influences. Melanomas are histologically classified into five distinct stages: common acquired and congenital nevi without dysplasia, dysplastic nevi, radial-growth phase (RGP) melanoma, vertical-growth phase (VGP) melanoma and metastatic melanoma (Fig.1). The first phenotypic change in melanocytes is the development of benign nevi, in which the activation of oncogenic pathways disrupts the control of growth in melanocytes, but the progression of nevus to cancer is rare, probably due to oncogene-induced cell senescence. Next cytologic atypia develops in dysplastic nevi, probably arising from preexisting benign nevi or as new lesions. At this stage of progression, cumulative mutations result in dysregulation of cell growth, DNA repair, and the susceptibility to cell death. Compared with RGP melanoma growing laterally and largely confined to the epidermis, VGP melanoma invades the upper layers of the epidermis and penetrates into the underlying dermis and subcutaneous tissue, where it forms expansible nodules of malignant cells. The transition from RGP to VGP melanoma is considered to be the crucial step in the evolution to malignant melanoma (Miller and Mihm 2006).

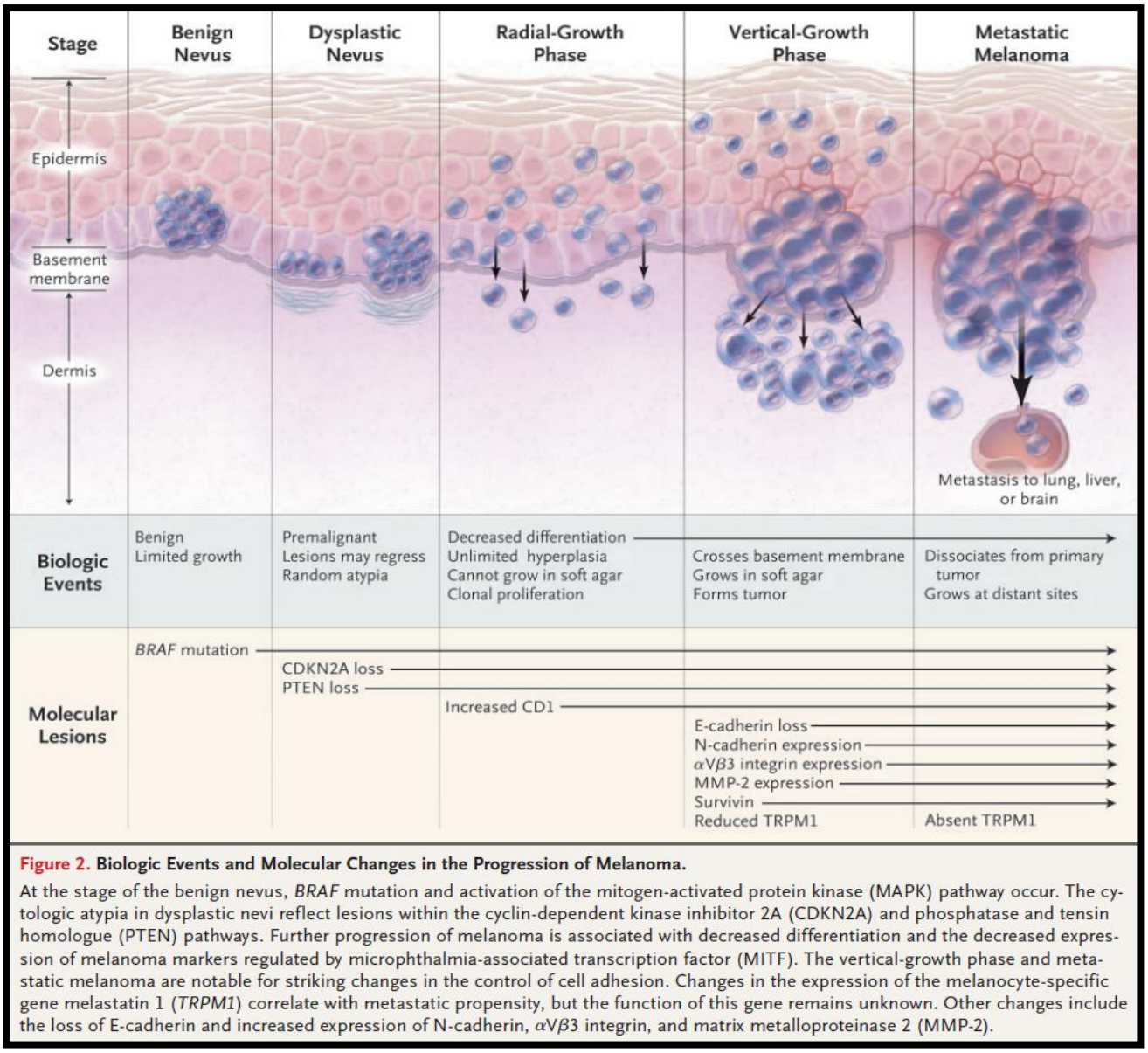



**Figure 2. Biologic Events and Molecular Changes in the Progression of Melanoma.**

At the stage of the benign nevus, *BRAF* mutation and activation of the mitogen-activated protein kinase (MAPK) pathway occur. The cytologic atypia in dysplastic nevi reflect lesions within the cyclin-dependent kinase inhibitor 2A (CDKN2A) and phosphatase and tensin homologue (PTEN) pathways. Further progression of melanoma is associated with decreased differentiation and the decreased expression of melanoma markers regulated by microphthalmia-associated transcription factor (MITF). The vertical-growth phase and metastatic melanoma are notable for striking changes in the control of cell adhesion. Changes in the expression of the melanocyte-specific gene melastatin 1 (*TRPM1*) correlate with metastatic propensity, but the function of this gene remains unknown. Other changes include the loss of E-cadherin and increased expression of N-cadherin, αVβ3 integrin, and matrix metalloproteinase 2 (MMP-2).

**Figure 1: Biologic Events and Molecular Changes in the Progression of Melanoma (Miller and Mihm 2006)**.

### 1.3.2 Signaling in melanoma

#### 1.3.2.1 The RAS/RAF/MEK/ERK pathway

The mitogen-activated protein kinase (MAPK) pathway is evolutionarily conserved. It is composed of different kinases linking extracellular signals to the machinery that controls fundamental cellular processes such as growth, proliferation, differentiation, migration and apoptosis.

A wide variety of receptors involved in growth and differentiation including receptor tyrosine kinases (RTKs), integrins, and ion channels are able to activate the MAPK pathway. The

receptors are linked by a set of adaptors (Shc, Grb2, Crk, etc.) to a guanine nucleotide exchange factor (SOS, C3G, etc.), which transduces the signal to small GTP-binding proteins (RAS, Rap1). Sequently they activate the core unit of the cascade, which is composed of Raf, MEK1/2 and ERK. D-type cyclins and various transcription factors are phosphorylated by an activated ERK dimer, and further regulate cell survival and proliferation (Pimienta and Pascual 2007; Raman, Chen et al. 2007; Shaul and Seger 2007). Abnormal activation of the MAPK pathway impairs the cell cycle control, which is essential for melanoma development. In melanoma the activation of this pathway is mainly due to somatic mutations of NRAS, which are associated with about 15% of melanomas, or BRAF, which are associated with about 50% of melanomas. These mutations appear in a mutually exclusive manner (Albino, Nanus et al. 1989; Brose, Volpe et al. 2002; Omholt, Platz et al. 2003).

#### 1.3.2.2 PI3K Pathway

Phosphatidylinositol-3 kinases (PI3Ks) is composed of a lipid kinase family characterized by their ability to phosphorylate an inositol ring 3'-OH group in inositol phospholipids to generate the second messenger phosphatidylinositol-3,4,5-trisphosphate (PIP3) and Phosphatidylinositol-4,5-bisphosphate (PIP2). RTK activation leads to production of PIP3 and PIP2 by PI3K at the inner side of the plasma membrane. AKT interacts with these phospholipids, which causes its translocation to the inner membrane, where it is phosphorylated and activated by phosphoinositide-dependent kinases 1/2 (PDK1/2). Activated AKT can phosphorylate numerous substrates that regulate cell survival, cell cycle progression and cellular growth (Bellacosa, Kumar et al. 2005; Madhunapantula and Robertson 2009). This signaling is inhibited by the tumor suppressor phosphate and tensin homologue (PTEN) (Keniry and Parsons 2008; Aguissa-Toure and Li 2012).

#### 1.3.2.3 AKT

Overexpression and hyperactivation of AKT, the V-Akt Murine Thymoma Viral Oncogene Homolog 3, are present at 60-70% of sporadic melanomas (Stahl, Sharma et al. 2004). Significant phosphorylation of AKT3 was registered in 17%, 43%, 49% and 77% of biopsies from normal nevi, dysplastic nevi, primary melanoma and melanoma metastases,

respectively. It´s a poor prognostic factor for patients with melanoma less than 1.5 mm thickness as the level of expression of phosphorylated AKT3 is increased (Dai, Martinka et al. 2005).

#### 1.3.2.4 PTEN

##### 1.3.2.4.1 Structure

The PTEN gene, also known as TGF-β-regulated and epithelial cell enriched phosphatase (TEP1), is a tumor suppressor gene, which is isolated and identified by three laboratories in 1997. The human genomic PTEN is located on chromosome 10q23.3. It consists of 9 exons and 8 introns, and encodes a 5.5 kb mRNA that defines a 403 amino-acid open reading frame. PTEN is a 53 kDa protein with high homology to tensin/auxilin and protein tyrosine phosphatases (PTPs) (Li and Sun 1997; Li, Yen et al. 1997; Steck, Pershouse et al. 1997; Lee, Yang et al. 1999; Waite and Eng 2002). Studies of the crystal structure of PTEN have revealed that the human PTEN protein is composed of four functional modules: (1) N-terminal residue of the phosphatase domain carrying the conserved phosphatase motif HCSSGSSR. This domain is highly homologous to the catalytic domains of tyrosine phosphatase and serine/threonine phosphatase, indicating that it has ability to dephosphorylate tyrosine and serine/threonine residues. Most of missense mutations involved in human tumors and Cowden syndrome have been found in the phosphatase domain (Maehama, Taylor et al. 2001); HTTP://www.hgmd.cf.ac.uk/ac/gene.php?gene_PTEN) and biochemical analysis have confirmed that such mutations lead to a robust decrease in the phosphatase activity (Han, Kato et al. 2000; Koul, Jasser et al. 2002); (2) C- terminal residue of the C2 domain combines with phospholipids in a $Ca^{2+}$ - independent manner, and by this PTEN is localized in cell membranes, where it´s able to mediate an signal transduction. One study indicated that the modulation of molecular interaction between the phosphatase and C2 domains by phosphorylation of the C- terminal tail of PTEN dictates the percentage of membrane-associated PTEN and the level of PIP3 (Rahdar, Inoue et al. 2009); (3) The PDZ protein- binding domain and two PEST sequences (rich in Proline (P), Glutamic acid (E),

Serine (S) and Threonine (T)) in the tail region consist of multiple phosphorylation sites (Ser-370, Ser-380, Thr-382, Thr-383, and Ser-385) indispensable for PTEN protein stability. In fact, C-terminal deletion and phosphorylation-resistant mutants are quite unstable and rapidly degraded in cells (Vazquez, Ramaswamy et al. 2000; Torres and Pulido 2001; Birle, Bottini et al. 2002; Miller, Lou et al. 2002).

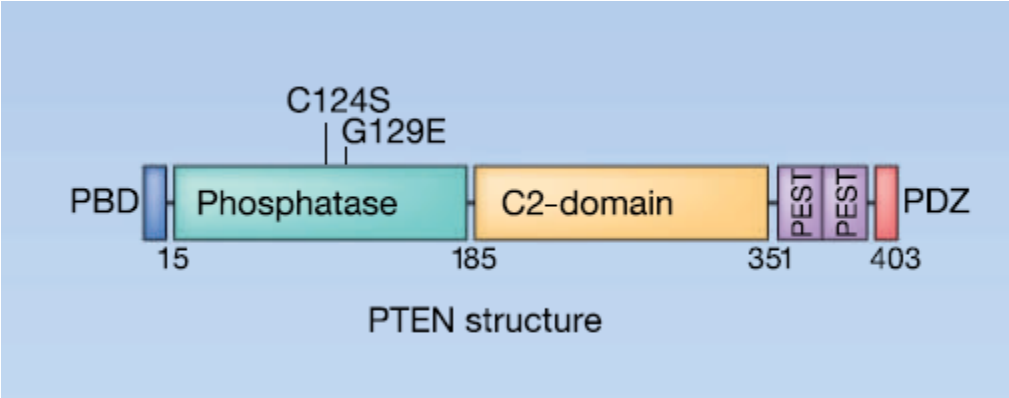



**Figure 2: PTEN protein structure.** PTEN protein is composed of an N-terminal PIP2-binding motif, a phosphatase domain, a C2 domain, a C-terminal tail containing PEST sequences, and a PDZ interaction motif at the end. PTEN's phosphatase activity is disrupted by naturally occurring mutations on the phosphatase domain: C124S mutation abrogates both lipid and protein phosphatase activity, and G129E mutation abrogates only lipid phosphatase but not protein phosphatase activity (Zhang and Yu 2010).

#### 1.3.2.4.2 Biological function

PTEN is a tumor suppressor gene. Studies over the past 10 years have revealed that a variety of cellular signal transduction pathways or molecules are regulated by PTEN, and form a complex network system to control proliferation, apoptosis, migration, adhesion and genetic stability. PTEN can dephosphorylate not only lipids but also proteins. Also, it can regulate signal transduction by both phosphatase- dependent and independent manners. PTEN is different from most tumor suppressor genes, and can function in the nucleus as well as in the cytoplasm. It has been shown that PTEN is associated with organelles such as the endoplasmic reticulum (ER), the mitochondria or the nucleus, and is secreted. PTEN activity can be modulated by mutations, epigenetic silencing, transcriptional repression, aberrant protein localization, and post-translational modifications. Also, its stability and function can be highly impacted by its precise intracellular localization (Tamguney and Stokoe 2007; Chetram and Hinton 2012).

The negative regulation on PI3K/AKT pathway by PTEN has been deeply studied. In the cytoplasm dephosphorylation of PIP3 to PIP2 by PTEN reverses the action of PI3K and hampers all downstream functions controlled by the AKT/mTOR axis, such as cycle progression, induction of cell death, transcription, translation, stimulation of angiogenesis, and stem cell self-renewal (Maehama and Dixon 1998). Additionally, cytoplasmic PTEN can regulate epidermal growth factor (EGF) signaling by inhibiting phosphorylation of SHC adaptor proteins, which in turn suppress activation of the MAPK pathway (Gu, Tamura et al. 1998). Finally, PTEN can also regulate the activity of focal adhesion kinases (FAKs) through dephosphorylation and other membrane channels to control cell migration, stretching and adhesion (Tamura, Gu et al. 1999).

Like other tumor suppressor genes, nuclear PTEN has important biological functions. The level of functional PTEN in the nucleus is regulated through dynamic changes in its ubiquitylation. PTEN mono-ubiquitination by the E3 ubiquitin- protein ligase NEDD4 controls its nuclear entry (Wang, Trotman et al. 2007). The promyelocytic leukemia protein-herpes virus-associated ubiquitin-specific protease (HAUSP) network controls PTEN deubiquitination and PTEN nuclear exclusion (Song, Carracedo et al. 2011). Nuclear PTEN can induce G0- G1 cell cycle arrest by reducing the level of cyclin D1, and inhibit tumor growth by regulating cellular senescence through anaphase promoting complex (APC)-CDH1-mediated protein degradation (Waite and Eng 2002; Leslie and Downes 2004). A study showed that PTEN can physically associate with centromeres to maintain centromeric stability. It is also shown that PTEN is involved in DNA-damage responses through upregulation of the transcription of Rad51, a key component of the homologous recombination system, which repairs DNA double-strand breaks (DSBs) (Leslie, Batty et al. 2008; Poliseno, Salmena et al. 2010). This guardian role of PTEN in maintaining chromosome structure and function seems to be independent of its lipid phosphatase activity (Shen, Balajee et al. 2007; Kim, Xu et al. 2011). Also, nuclear PTEN has a longer half-life and is more stable than its cytoplasmic counterpart; therefore the former possibly plays a greater role in suppressing tumor formation.

### 1.3.2.4.3 PTEN in melanoma

As a tumor suppressor, PTEN inhibits melanoma cell growth (Weng, Smith et al. 1999) by increasing susceptibility of cells to apoptosis (Lu, Lin et al. 1999). Ectopically expressed PTEN protein can inhibit invasion and induce apoptosis in melanoma cells (Stewart, Mhashilkar et al. 2002; Stahl, Cheung et al. 2003). PTEN loss and $BRAF^{V600E}$ have been shown to cooperate in promoting metastatic melanoma development (Dankort, Curley et al. 2009).

Cutaneous melanomas are characterized with a significant frequency of PTEN mutations. Mutations/deletions in the PTEN gene were found in 5-15% in primary melanomas and metastases, while the frequency of PTEN mutations increases up to 30-40% in melanoma cell lines (Tsao, Zhang et al. 1998; Yin and Shen 2008). Chromosome 10 deletion, endogenous microRNAs and promoter methylation are mainly responsible for decreased PTEN expression in melanoma patient tumor material (Mertens, Johansson et al. 1997; Wu, Goel et al. 2003; Mirmohammadsadegh, Marini et al. 2006). Moreover, intragenic polymorphisms in introns have been reported to regulate PTEN expression in melanomas (Tsao, Zhang et al. 1998; Yin and Shen 2008). Amino acid altering mutations (P95S, F154L, L325F) induced by Ultraviolet radiation has been shown to impair PTEN function, which promotes the development of early melanomas in xeroderma pigmentosum (Wang, Digiovanna et al. 2009).

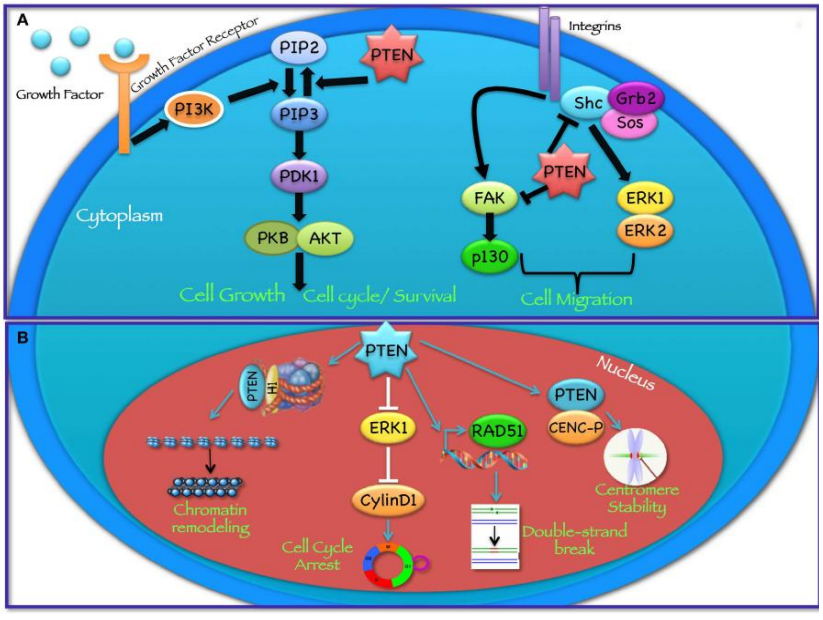


**Figure 3: Cytoplasmic and nuclear PTEN functions.** PTEN regulates a wide spectrum of biological functions, at least in part determined by its subcellular localization. **(A)** In the cytoplasm dephosphorylation of PIP3 to PIP2 by PTEN reverses the action of PI3K and hampers all downstream functions controlled by the AKT/mTOR axis, such as cycle progression, induction of cell death, transcription, translation, stimulation of angiogenesis, and stem cell self-renewal. In addition, PTEN affects cell migration by modulating complex pathways through its protein phosphatase activity directed against FAK and SHC. **(B)** In the nucleus, PTEN can maintain genomic integrity, repair DNA DSBs, control homologous recombination, and promote ubiquitin-dependent degradation of oncoproteins such as Polo-Like Kinase 1 (PLK1) and Aurora Kinase (AURK). In addition, PTEN modulates ERK phosphorylation and cyclin D1 levels to control cell-cycle progression and binds to histone H1 to regulate chromatin remodeling (Milella, Falcone et al. 2015).

#### 1.3.2.5 The insulin-like growth factor system

The insulin-like growth factor 1 (IGF-1) pathway plays relevant roles in regulating cellular proliferation and apoptosis regarding nourishment. Additionally it also regulates energy metabolism, body size, longevity and various organ-specific functions (Jones and Clemmons 1995; Nakae, Kido et al. 2001; Baserga, Peruzzi et al. 2003). The ability of the IGF pathway to control rates of cell renewal has led to interest in the relevance of this regulatory system to both ageing and neoplasia (Holly 1998; Burroughs, Dunn et al. 1999). It has been shown that proliferation and survival of cells propagated in tissue culture can be increased by IGF-1 stimulation (Jones and Clemmons 1995). Several model systems have confirmed that the activation of insulin-like growth factor 1 receptor (IGF-1R) stimulates proliferation and promotes metastasis of cancer cells, either in relation to higher levels of circulating IGF-1 in the host or to autocrine production of ligands by neoplastic cells (Khandwala, McCutcheon et al. 2000).

The IGF system consists of multiple receptors and ligands. There are three ligands (IGF-1, IGF-2 and insulin), four receptors and at least six high-affinity binding proteins and binding protein proteases.

##### 1.3.2.5.1 Ligand and binding protein

IGF-1 and -2 share 60% homology with proinsulin (LeRoith and Roberts 2003). IGFs and insulin bind to their cognate receptor with high affinity and inversely to the noncognate receptor with lower affinity. Unlike to insulin, IGFs can be synthesized widely by many cell types and are secreted immediately rather than stored intracellularly, while circulating IGF-1

is principally produced in the liver regarding growth hormones (D'Ercole, Stiles et al. 1984). In the circulation, the ligands are mostly bound to a family of high affinity binding proteins, the IGF binding proteins (IGFBPs). They are produced by the liver and range in size from 22 to 31 kDa. More than 95% of circulating IGF-1 is complexed with IGFBP-3 and the acid labile subunit (ALS) (Jones and Clemmons 1995). Although the function of IGFBP varies in relation to different cell types, they principally protect IGFs from degradation by proteases and also inhibit their interactions with the IGF-1R. It has been shown that IGFBP-1, IGFBP-3 and IGFBP-5 have function in ligand independent manner. Among them IGFBP-3 has been shown to promote IGF-independent apoptosis and to interact with the retinoid X receptor in the nucleus to influence gene transcription (Liu, Lee et al. 2000; Franklin, Ferry et al. 2003). Various protease enzymes including prostate-specific antigen (PSA) serve to favor ligand binding to the IGF-1R by cleaving IGFBPs (Rajaram, Baylink et al. 1997; Maile and Holly 1999).

#### 1.3.2.5.2 Receptor

The IGF receptor family consists of IGF-1R, the type 2 IGF receptor (IGF-2R) and the insulin receptor (IR). The IGF-1R is a tyrosine kinase receptor and is activated by IGF-1, IGF-2 and by insulin at supraphysiological concentrations. In humans, the IGF1R gene is located at the distal end of chromosome 15, and codes a single polypeptide 1367 amino acid long. After removal of the 30 amino acid signal peptide, an endopeptidase cleaves the remaining 1337 amino acids at the Arg-Lys-Arg-Arg site between residues 707 and 710. This proteolytic digestion releases the α- and ß-subunits (Ullrich, Gray et al. 1986). These subunits are connected by means of disulfide bonds to give rise to the mature tetrameric receptor: two α-subunits is entirely extracellular and responsible for specific ligand binding whereas the two ß-subunits span the plasma membrane and encompass an intracellular domain, which serves to the initiation of signal transduction cascades (Ward and Garrett 2004).

The IGF-2R gene encodes for a monomeric transmembrane protein lacking a tyrosine kinase domain and has no capability to transduce signaling. It acts as a negative regulator of IGF activity though endocytosis and degradation of IGF-2 (Nolan, Kyle et al. 1990). The IGF-2R

acts as the mannose-6-phosphate (M6P) receptor to bind lysosomal enzymes and other M6P-containing proteins for transfer to the lysosome (Kornfeld 1992; Hille-Rehfeld 1995).

There are two isoforms of IR generated by alternative splicing of exon 11. IR-A is more highly expressed in fetal tissue (Frasca, Pandini et al. 1999). IR-A and IR-B have similar affinities for insulin, but IR-A exhibits higher affinity for IGF-2 than IR-B (Yamaguchi, Flier et al. 1993).

The IGF-1R is closely related to IR. They have a similar tetrameric structure, which is composed of two polypeptide chains, each with an extracellular α-subunit and β-subunit. At the amino acid level the IGF-1R is 70% homologous to the IR, especially with 84% similarity in the tyrosine kinase domain but in C-terminus only 44%, which indicates that there is difference in signaling and function for both. (Ullrich, Gray et al. 1986).

Also, there is hybrid IGF-1R/IR assembled with one αβ chain of IGF-1R and one of IR in cells (Frasca, Pandini et al. 1999; Pandini, Vigneri et al. 1999). The IGF-1R/IR-B hybrids exhibit affinity to IGF-1 as same as IGF-1R while IGF-1R/IR-A hybrid receptors bind to IGF2 with high affinity. Thus, preclinical data have shown that IR plays an important role for regulation of IGF action, either as a hybrid or homo IR-A receptor (Morrione, Valentinis et al. 1997; Kalli, Falowo et al. 2002). It was considered that the IGF-1R controls mitogenesis, while the IR functions in metabolism and glucose homeostasis. However, studies over the past several years have revealed that they play distinct and overlapping roles. IR can also function in several aspects of breast cancer biology, either as a hybrid or homoreceptor (Sciacca, Mineo et al. 2002).

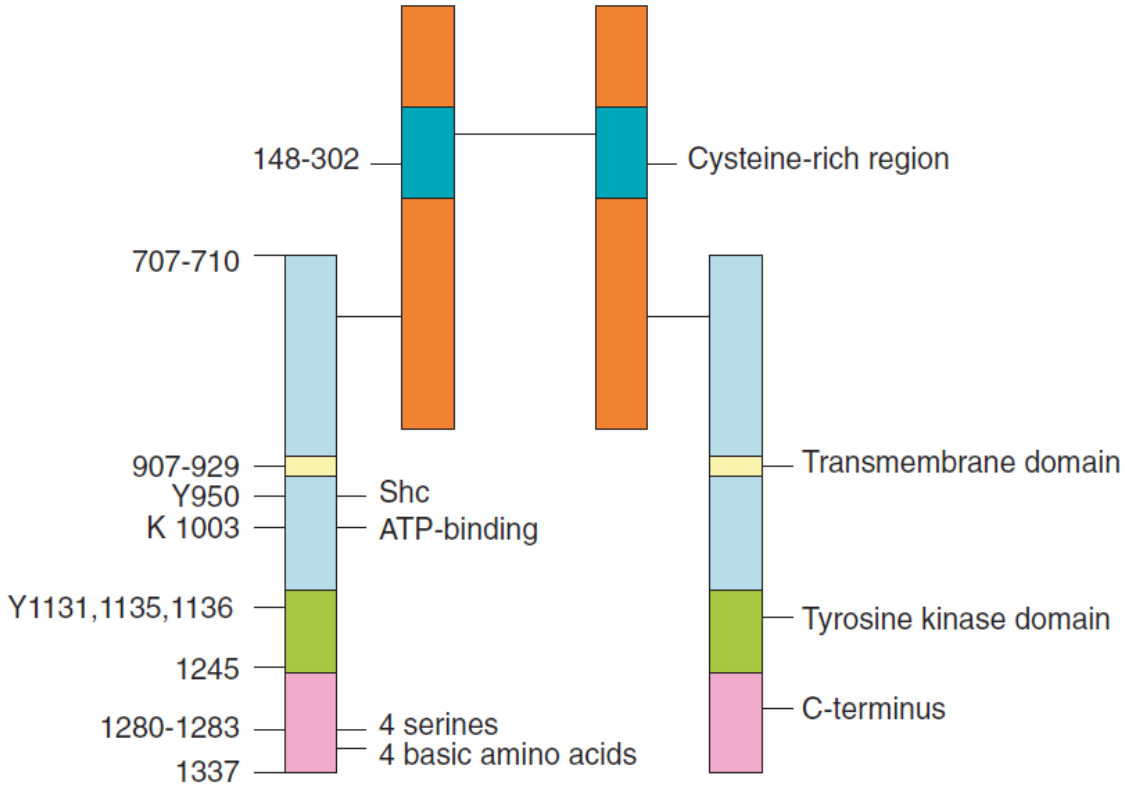



**Figure 4 Schematic representation of the IGF-1R.** IGF-1R is a heterotetrameric molecule, composed of two identical polypeptide chains, each with a α- and β-chains linked by disulfide bonds. The α-chains are extracellular and responsible for specifically binding of the growth factors, while the β-chains contain a short extracellular region, a transmembrane region, an intracellular tyrosine kinase domain, and a C-terminal tail. Key residues of the IGF-1R are shown on left side. The amino acid numbers in this figure are those of the human receptor after removal of the 30 amino acid signal peptide (Baserga 2005).

### 1.3.2.5.3 IGF-1R signaling

Binding of IGFs to IGF-1R results in a conformational change in the receptor, which leads to an auto-phosphorylation of tyrosines 1131, 1135 and 1136 in the kinase domain, juxtamembrane tyrosines and C-terminal serines (Kato, Faria et al. 1994; Parvaresch, Yesilkaya et al. 2002). These phosphorylation events recruits signaling intermediates including insulin-receptor substrate (IRS)- 1 and -2, Shc, Grb10, and 14-3-3e (Myers, Grammer et al. 1994; White and Kahn 1994; Craparo, O'Neill et al. 1995; Craparo, Freund et al. 1997), and in turn results in activation of distinct signaling pathways (Ricketts, Rose et al. 1996; Nguyen, Wang et al. 2000). The first detailed study of IGF-1R regulated pathway focused on the PI3K/AKT pathway. The phosphorylated IGF-1R can recruit PI3K via SH2 domains of the adaptor protein p85 through phosphorylated IRS to the cell membrane, where

PI3K is activated and leads to synthesis of membrane-associated PIP3. This leads to activation of PDKs, and in turn a spectrum of downstream protein kinases, including AKT, p70RSK and protein kinase C (PKC) are phosphorylated and activated (Vanhaesebroeck and Alessi 2000; Manning and Cantley 2007). Through these effectors IGF-1R controls a wide range of effects, including mitogenesis, proliferation, cell cycle control and inhibition of apoptosis (Vanhaesebroeck and Alessi 2000). The second major principal signaling pathway is MAPK pathway. Recruitment of the SOS to IRS-1 or Shc, via the SH2 domain of the Grb2 adaptor protein leads to activation of the small G protein RAS, which in turn activates the protein serine kinase Raf and the MAPK pathway (Davis 1995). This pathway mediates cell proliferation and differentiation. In addition to these two principal pathways mediated via tyrosine-phosphorylated IRS and/or Shc, a spectrum of proteins can interact directly with the receptors and act independently or in synergy with IRS and Shc. For example, the IGF-1R can interact directly with14-3-3 proteins, causing the translocation of c-Raf to the mitochondria and by this protects cells from apoptosis (Peruzzi, Prisco et al. 1999). Moreover, phospholipase C and PKC, the signal transducers and activators of transcription (STATs), and their negative regulators the suppressors of cytokine signaling (SOCS) can also be activated directly by IGF-1R (Chen, Sadowski et al. 1997; Dey, Spence et al. 1998; Zong, Zeng et al. 1998). Unlike the widespread expression of the IGF-1R, IR expression is restricted to the liver, adipose tissue and muscle. Activation of IR by binding Insulin leads to glucose uptake and inhibition of gluconeogenesis in the liver (Olefsky 1990; White and Kahn 1994). Although IGF-1R and IR signaling have a few common and overlapped functions, they differ in many aspects, including the different patterns of receptor expression, kinetics of ligand binding, recruitment of signaling intermediates and effects on gene expression (De Meyts, Christoffersen et al. 1995; Siddle, Urso et al. 2001).

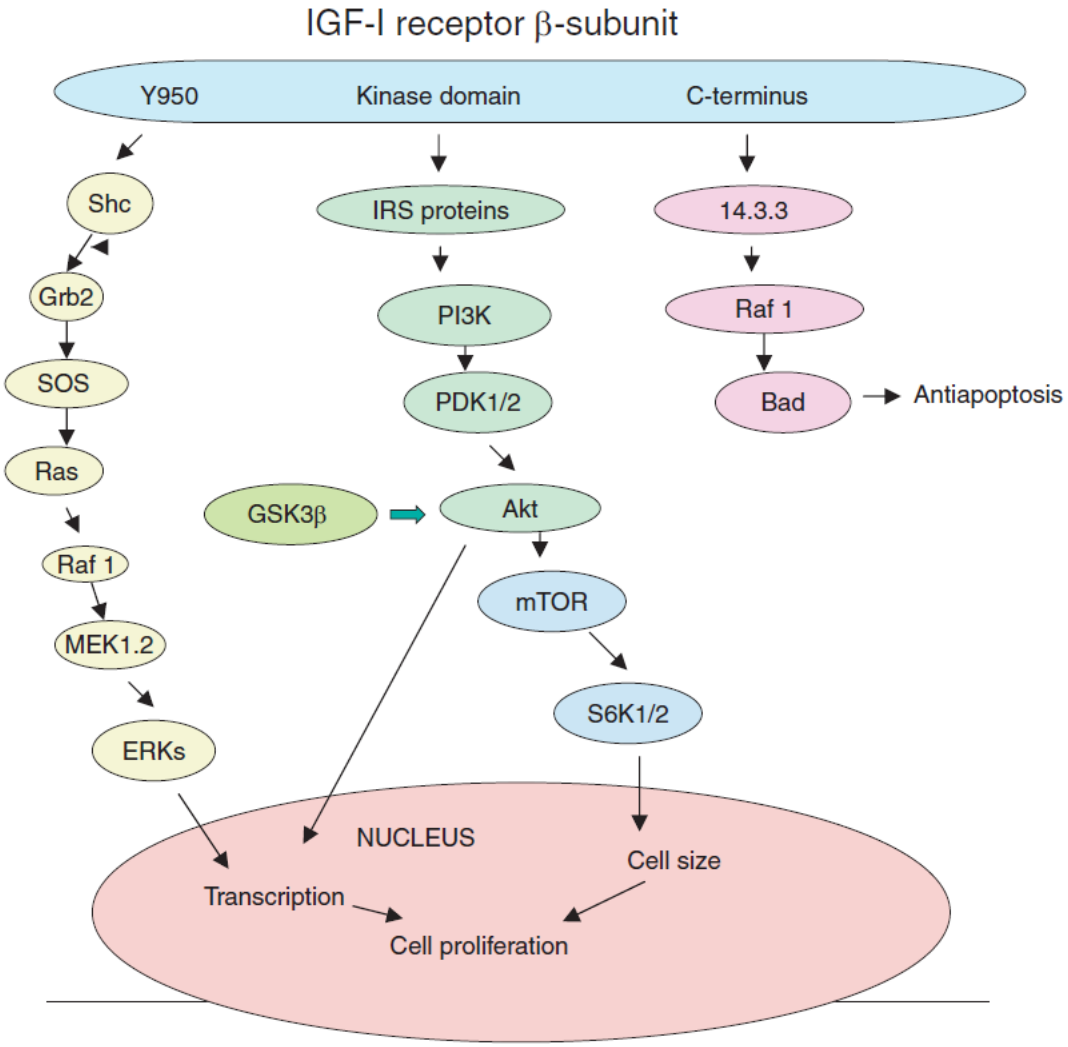



**Figure 5 Signaling pathways of the IGF-1R.** Schematic diagram of the three main pathways originating from the IGF-1R. Binding of IGF-1/2 to IGF-1R leads to autophosphorylation of its tyrosine kinase domain and subsequently various cytoplasmic mediators are activated. Particularly important pathways are the RAS-Raf-MEK-ERK1/2 and the PDK-AKT-mTOR signaling systems. Also, direct interaction of IGF-1R with 14-3-3 proteins causes the translocation of c-Raf to the mitochondria. Though these mediators IGF-1R controls a wide range of effects, including mitogenesis, proliferation, cell cycle control and inhibition of apoptosis (Baserga 2005).

### 1.3.2.5.4 IGF-1R in melanoma

IGF-1R is overexpressed in melanoma and is correlated with melanoma progression (Kanter-Lewensohn, Girnita et al. 2000). Previous studies have demonstrated that IGF-1 stimulation promotes proliferation of early-stage melanoma cells, but not metastatic melanoma cells (Satyamoorthy, Li et al. 2001). A more recent study showed that IGF-1 inhibits tumor-necrosis-factor-related apoptosis-inducing ligand (TRAIL)-induced apoptosis

by inducing expression of BCL2, BCL-xl and surviving (Hilmi, Larribere et al. 2008). Downregulation of IGF-1R expression or activity causes growth arrest and apoptosis (Kanter-Lewensohn, Dricu et al. 1998; Kanter-Lewensohn, Girnita et al. 2000; Maloney, McLaughlin et al. 2003). Also, several studies have shown that human melanoma cells expressing $BRAF^{V600E}$ are susceptible to IGF-1R targeting (Maloney, McLaughlin et al. 2003; Yeh, Bohula et al. 2006; Macaulay, Middleton et al. 2013). Recently, the importance of IGF-1R expression and activity was stressed again in therapy-resistance of malignant melanoma against the $BRAF^{V600E}$ inhibitor PLX4032 (Villanueva, Vultur et al. 2010). Interestingly, the persistent IGF-1R activity in melanoma cells resistant to the BRAF inhibitor was not mediated by an autocrine IGF-1 mediated signalling (Villanueva, Vultur et al. 2010). In line with this, a recent study showed that fibroblast-derived IGF-1 could not mediate resistance towards BRAF inhibition (Straussman, Morikawa et al. 2012). This suggests that the enhanced expression and activity of IGF-1R signalling in melanoma cells is regulated by melanoma cell intrinsic regulatory mechanisms.

### 1.4 Aim of the project

Among RTKs, the IGF-1R is one of the most important players in cancer development. In melanoma, the IGF-1R is overexpressed compared with benign naevi (Kanter-Lewensohn, Girnita et al. 2000). IGF-1R antisense oligonucleotides have been shown to inhibit melanoma cell survival *in vitro* and the growth of FO-1 melanoma xenografts in nude mice (Resnicoff, Coppola et al. 1994). Knockdown of IGF-1R is capable of inducing significant survival inhibition and enhancement of apoptosis, regardless of BRAF mutation status (Maloney, McLaughlin et al. 2003; Yeh, Bohula et al. 2006; Macaulay, Middleton et al. 2013). The importance of IGF-1R expression and activity was stressed again in therapy-resistance of malignant melanoma against the $BRAF^{V600E}$ inhibitor PLX4032 (Villanueva, Vultur et al. 2010). These findings suggest that the IGF-1R is an attractive target for melanoma treatment. However, in clinical settings, no clear mechanism of aberrant IGF-1R could be recognized. The large increases in receptor number are rarely associated with gene amplification. Therefore, a deeper understanding of the molecular mechanisms regulating IGF-1R

expression and activity in melanoma cells is indispensable.

In this study we analyzed the regulatory mechanisms involved in enhancing IGF-1R expression and activity in melanoma cells and the impact on therapy resistance of melanoma cells. We focused on the impact of the MAPK- and PI3K/AKT pathways on IGF-1R expression and activity.

# 2 Materials and Methods

## 2.1 Materials

### 2.1.1 Cell lines

| Cell lines | Obtained from | Described in | Mutation |
|---|---|---|---|
| 451Lu | Meenhard Herlyn, Wistar Institut, Philadelphia, USA | Andalib, Lawry *et al.* 1997 | $BRAF^{V600E}$<br>NRAS WT<br>PTEN nd |
| SKMEL19 | Meenhard Herlyn, Wistar Institut, Philadelphia, USA | Carey, Takahashi *et al*. 1976 | $BRAF^{V600E}$<br>NRAS WT<br>PTEN WT |
| 1205Lu | Meenhard Herlyn, Wistar Institut, Philadelphia, USA | Andalib, Lawry *et al*. 1997 | $BRAF^{V600E}$<br>NRAS WT<br>PTEN Mu/ Hem Del |
| WM3734 | Meenhard Herlyn, Wistar Institut, Philadelphia, USA | The Wistar Institute | $BRAF^{V600E}$<br>NRAS WT<br>PTEN Hom Del |
| MDAMB435 | ATCC | Rae, Creighton *et al*. 2007 | $BRAF^{V600E}$<br>NRAS WT<br>PTEN WT |
| Malme-3M | ATCC | Fogh, Wright *et al.* 1977 | $BRAF^{V600E}$<br>NRAS WT<br>PTEN WT |
| M14 | ATCC | Paraiso, Xiang *et al.* 2011 | $BRAF^{V600E}$<br>NRAS WT<br>PTEN WT |
| WM35 | Meenhard Herlyn, Wistar Institut, Philadelphia, USA | Paraiso, Xiang *et al.* 2011 | BRAF WT<br>$NRAS^{Q61R}$<br>PTEN WT |
| WM115 | Meenhard Herlyn, Wistar Institut, Philadelphia, USA | The Wistar Institute | BRAF WT<br>NRAS WT<br>PTEN Hem Del |

| | | | |
|---|---|---|---|
| WM793 | Meenhard Herlyn, Wistar Institut, Philadelphia, USA | Paraiso, Xiang *et al.* 2011 | $BRAF^{V600E}$<br>NRAS WT<br>PTEN Mu/Hem Del |
| WM266 | Meenhard Herlyn, Wistar Institut, Philadelphia, USA | The Wistar Institute | $BRAF^{V600E}$<br>NRAS WT<br>PTEN Hem Del |
| IGR39 | C. Garbe (Department of Dermatology, University of Tübingen, Germany | Aubert, Chirieceanu *et al.* 1976 | $BRAF^{V600E}$<br>NRAS WT<br>PTEN Hem Del |
| A375 | ATCC | Giard, Aaronson *et al*. 1973 | $BRAF^{V600E}$<br>NRAS WT<br>PTEN WT |
| SKMel147 | | Wang, Lee *et al*. 2012 | BRAF WT<br>$NRAS^{Q61R}$<br>PTEN nd |
| SKMel30 | | Carey *et al.*, 1976 | $BRAF^{D287H}$<br>$NRAS^{Q61K}$<br>PTEN WT |
| MV3 | Meenhard Herlyn, Wistar Institut, Philadelphia, USA | van Muijen, Jansen *et al*. 1991 | BRAF WT<br>$NRAS^{Q61R}$<br>PTEN WT |
| M1617 | Meenhard Herlyn, Wistar Institut, Philadelphia, USA | Villanueva, Vultur *et al.* 2010 | $BRAF^{V600E}$<br>NRAS nd<br>PTEN WT |

* WT: wild-type; Mu: mutated; Hem Del: hemizygous deletion; nd: no data available

| **Cell line** | **Cell type** | **Prepared from** |
|---|---|---|
| FF (foreskin fibroblasts) | Primary fibroblasts | Foreskin |
| Melanocytes | Primary melanocytes | Foreskin |

### 2.1.2 Antibodies

| Antibody | Source | Catalog-number | Dilution | Distributor |
|---|---|---|---|---|
| ß-Actin | Rabbit | #4967 | 1:1000 (WB) | Cell Signaling (NEB), Frankfurt a. M. |
| AKT3 | Rabbit | #3788 | 1:1000 (WB) | Cell Signaling (NEB), Frankfurt a. M. |
| AKT | Rabbit | #4685 | 1:1000 (WB) | Cell Signaling (NEB), Frankfurt a. M. |
| phospho-AKT (Ser473) | Rabbit | #4060 | 1:1000 (WB) | Cell Signaling (NEB), Frankfurt a. M. |
| ERK | Rabbit | #9102 | 1:1000 (WB) | Cell Signaling (NEB), Frankfurt a. M. |
| phospho-ERK (Thr202/Thr204) | Rabbit | #9101 | 1:1000 (WB) 1:100(FC) | Cell Signaling (NEB), Frankfurt a. M. |
| pIGF-1R | Rabbit | #3918 | 1:1000 (WB) | Cell Signaling (NEB), Frankfurt a. M. |
| IGF-1Rß | Rabbit | #3027 | 1:1000 (WB) | Cell Signaling (NEB), Frankfurt a. M. |

| | | | | |
|---|---|---|---|---|
| IGF-1Rß | Rabbit | sc713 | IHC | Santa Cruz Biotechnology, Heidelberg |
| IRS1 | Rabbit | #3407 | 1:1000 (WB) | Cell Signaling (NEB), Frankfurt a. M. |
| IRS2 | Rabbit | #3089 | 1:1000 (WB) | Cell Signaling (NEB), Frankfurt a. M. |
| B-Raf | Mouse | sc5284 | 1:500(WB) | Santa Cruz Biotechnology, Heidelberg |
| PTEN | Rabbit | # 9559 | 1:1000 (WB) (IHC) | Cell Signaling (NEB), Frankfurt a. M. |
| IgG HRP-linked | anti-Rabbit | #7074 | 1:2500 | Cell Signaling (NEB), Frankfurt a. M. |
| IgG HRP-linked | anti-Mouse | #7076 | 1:2500 | Cell Signaling (NEB), Frankfurt a. M. |
| IGF-1R α | Mouse | 555999 | (FC) | BD, Pharmingen, Heidelberg |
| IgG1 | Mouse | 555749 | (FC) | BD, Pharmingen, Heidelberg |

| | | | | |
|---|---|---|---|---|
| APC conjugated anti-mouse IgG1 | Rabbit | 560089 | 1:100(FC) | BD, Pharmingen, Heidelberg |
| APC conjugated anti-rabbit IgG1 | Mouse | #5366 | 1:100(FC) | Cell Signaling (NEB), Frankfurt a. M. |
| C3 conjugated anti rabbit igG1 | Donkey | | (IHC) | Dianova GmbH,Hamburg |
| C5 conjugated anti mouse igG1 | Donkey | | (IHC) | Dianova GmbH,Hamburg |

* WB: Western Blot; IHC: Immunohistochemistry; FC: Flow Cytometry

### 2.1.3 Materials and Kits

| Material | Distributor |
|---|---|
| PVDF Membranes | Roche, Mannheim |
| Biocoat: Collagen I Coated Cultureware | BD Biosciences, Heidelberg |
| Nucleospin RNAII Kit | Macherey&Nagel, Düren |
| X-ray Film Kodak X-OMAT | Eastman Kodak Company, Rochester |
| Superscript II Reverse Transkriptase | Invitrogen, Darmstadt |
| SYBR Green Real-time PCR Mix | BioRad, München; Roche, Mannheim |
| Lipofectamin 2000 Transfection Reagent | Invitrogen, Darmstadt |
| Lipofectamin RNAimax Transfection Reagent | Invitrogen, Darmstadt |
| Whatman 3mm-Paper | Roth, Karlsruhe |
| QIAquick DNA Gel Extraction Kit | Qiagen, Hilden |
| Amersham ECL Prime Wester Blotting Detection Reagent | GE healthcare life science, Schwerte |
| Pierce ECL Western Blotting Substrate | Thermo Fisher Scientific, Bonn |
| Competent Ecoli | Clontech,Saint-Germain-en-Laye, France |
| Protein Assay Kits | Bio-Rad, München |

### 2.1.4 Chemicals

| Substance | Distributor |
|---|---|

| | |
|---|---|
| Fetal Calf Serum (FBS) | Biochem Berlin |
| Penicillin/ Streptomycin | Gibco, Schwerte |
| Puromycin | Sigma Aldrich Chemie GmbH, Taufkirchen |
| Gentamycin | Gibco, Schwerte |
| OPTIMEM Medium | Gibco, Schwerte |
| DMEM (dulbecco's modified eagle's medium) | Sigma Aldrich Chemie GmbH, Taufkirchen |
| RPMI | Gibco, Schwerte |
| Polybrene | Sigma Aldrich Chemie GmbH, Taufkirchen |
| 4-Methylumbelliferyl Heptanoate (MUH) | Sigma Aldrich Chemie GmbH, Taufkirchen |
| Phosphate Buffered Saline (PBS) | Sigma Aldrich Chemie GmbH, Taufkirchen |
| Propidium Iodide (PI) | Sigma Aldrich Chemie GmbH, Taufkirchen |
| 5x Passive Lysis Buffer | Promega, Mannheim |
| Agar Noble | Difco, Heidelberg |
| PLX4032 | LC Laboratories, Woburn,USA |
| AZD 6244 | Absource Diagnostics GmbH, München |
| Cell Lysis Buffer | Cell Signaling (NEB), Frankfurt a. M. |
| Bovine Serum Albumin (BSA) | Roth, Karlsruhe |
| Polyacrylamide | Roth, Karlsruhe |
| No Fat Milk | Cell Signaling (NEB), Frankfurt a. M. |
| Ampicillin | Sigma Aldrich Chemie GmbH, Taufkirchen |
| Gelred Nucleic Acid Gel Stain | Biotium, Köln |
| Para-formaldehyd (PFA) | Sigma Aldrich Chemie GmbH, Taufkirchen |

| | |
|---|---|
| Picropodophyllin (PPP) | Calbiochem, Darmstadt |
| PI-103 | Calbiochem, Darmstadt |
| Ipatasertib (AKTi) | Selleck chemicals Müchen |
| BKM | Selleck chemicals Müchen |
| GDC0941 | Selleck chemicals Müchen |
| Recombinant Human IGF-1 | R&D Systems GmbH, Wiesbaden-Nordenstadt |
| Doxycyclin | Sigma Aldrich Chemie GmbH, Taufkirchen |
| BES | Calbiochem, Darmstadt |
| RNase | Fermentas, St. Leon-Rot |
| Ethylene Diamine Tetraacetic Acid(EDTA) | Sigma Aldrich Chemie GmbH, Taufkirchen |
| YO-PRO-1 | Invitrogen, Darmstadt |
| Cycloheximide (CHX) | Sigma Aldrich Chemie GmbH, Taufkirchen |
| HMB45 | DAKO, |
| G418 | Sigma Aldrich Chemie GmbH, Taufkirchen and DAKO |

### 2.1.5 Devices

| **Device** | **Type/Company** |
|---|---|
| Vacuum Pump System | IV-500 Vacuset Multi-Tip Kit (Inotech) |
| Blot Equipment | Trans-Blot SD Semi-Dry Transfer Cell (Bio-Rad) |
| $CO_2$ Incubator | HERAcell 240 $CO_2$ Incubator<br>BBD 6220 ; T6 function line (Heraeus) |
| Electrophoresis Chamber | Mini-PROTEAN (Bio-Rad) |
| Heat Sealing Machine | Folio FS 3602 (Severin) |
| Heating Block | Dry-Block DB-2D (Techne) |
| Camera | E-300 (Olympus) |
| Refrigerator | FKS 500 (Liebherr); HFU 586 (Heraeus) |

| | |
|---|---|
| Magnetic Stirrer | RCT basic (IKA Labortechnik) |
| Microscope | CK 40 (Olympus) ; IX50 (Olympus) |
| Microwave | MW600 (Bosch) |
| Power Supply | Power Supply EPS 200 (Pharmacia Biotech); Power Pac 300 (BioRad) |
| pH-meter | Knick pH-Meter 761 (Calimatic) |
| Spectrophotometer | Smartspec plus (Biorad) |
| Microplate reader | TriStar LB 941 (Berthold Technologies) |
| Realtime PCR System | iCycler (Biorad); LC480 (Kalli, Falowo et al.) |
| Roll Mixer | RM5 (Fröbel Labortechnik) |
| X-ray Cassette | Röntgenkassette (Dr. Goos-Suprema |
| Incubator Shaker | HT (Infors) |
| Shaker | HS250 (IKA Labortechnik) |
| Safety Cabinets | Herasafe HS18 (Heraeus); MRF-B (Steag) |
| Thermocycler | PTC200 (MJ Research) |
| Ultra Centrifuge | 10SW Rotor (Sorvall) |
| Vacuum Pump | N180.3 (KNF Neuberger) |
| Vortexer | Vortex-Genie 2 (Scientific Industries) |
| Scale | LC 821 (Sartorius); Kern 770 (Kern) |
| Water Bath | 1083 (Gesellschaft für Labortechnik) |
| Centrifuge | Biofuge pico (Heraeus)<br>Biofuge 13 (Heraeus)<br>Megafuge 1.0R (Heraeus)<br>Varifuge 3.0R (Heraeus)<br>Biofuge fresco (Heraeus)<br>ZK 401 (Hermle) |
| Bacterium Incubator | HT (Infors) |
| Flow Cytometer | BD LSRII (BD Biosciences) |
| Gel Documentation | EpiChem3 Darkroom (UVP BioImaging Systems) |
| Confocal Microscope | TCS SP (Leica) |

| | |
|---|---|
| Fluorescence Microscope | AXIO IMAGER.A2 (Zeiss) |

### 2.1.6 Software

| Programm | Company |
|---|---|
| Acrobat Reader | Adobe |
| Word | Microsoft |
| Excel | Microsoft |
| Powerpoint | Microsoft |
| Photoshop | Adobe |
| Graphpad Prism Version 5.01 | GraphPad |
| ICycler iQ | Bio-Rad |
| LC480 | Roche |
| Microwin 2000 4.41 | Microtek |
| Confocal Software 2.61 | Leica |
| Scion Image | Scion Corporation |
| BD FACS DIVA | BD Biosciences |
| LEICA TCSSP | Leica |
| FCS EXPRESS | De Novo software |

### 2.1.7 Oligonucleotides

| Gene | Sequence |
|---|---|
| siControl | Sense 5’-acaacauucauauagcugccccc-3’ |
| | Anti sense 5’-gggggcagcuauaugaauguugu-3’ |
| siAKT3 | Sense 5’-gcucauuacuaggauauaatt-3’ |
| | Anti sense 5’-uuauauccuaugaauga cca-3’ |
| siBRAF | Sense 5’-ggagguguggaauaucaaatt-3’ |
| | Anti sense 5’-uuugauauuccacaccucctc-3’ |
| siIGF-1R | Sense 5’-gcaugguagccgaagauuutt-3’ |
| | Anti sense 5’-aaaucuucggcuaccaugcaa-3’ |

| Gene | PCR primer Sequence |
|---|---|
| GAPDH | Forward 5’-CTGACTTCAACAGCGACACC-3’ |

| | |
|---|---|
| | Reverse 5’-TAGCCAAATTCGTTGTCATACC-3’ |
| IGF-1R | Forward 5’-GCCGACGAGTGGAGAAATC-3’ |
| | Reverse 5’-GAGGTAGCCCTCGATCACC-3’ |
| 18S rRNA | Forward 5’-CGGCTACCACATCCAAGGAA-3’ |
| | Reverse 5’-GCTGGAATTACCGCGGCT-3’ |

### 2.1.8 Plasmids

#### 2.1.8.1 pMD2.G

Envelope plasmid used for lentivirus production. Mammalian expression of Vesicular Stomatitis Indiana Virus protein G (VSV-G) driven by the cytomegalovirus (CMV) promoter. It contains ampicillin resistance in bacteria. pMD2.G was a gift from Didier Trono at the Swiss Federal Institute of Technology Lausanne (Addgene plasmid # 12259).

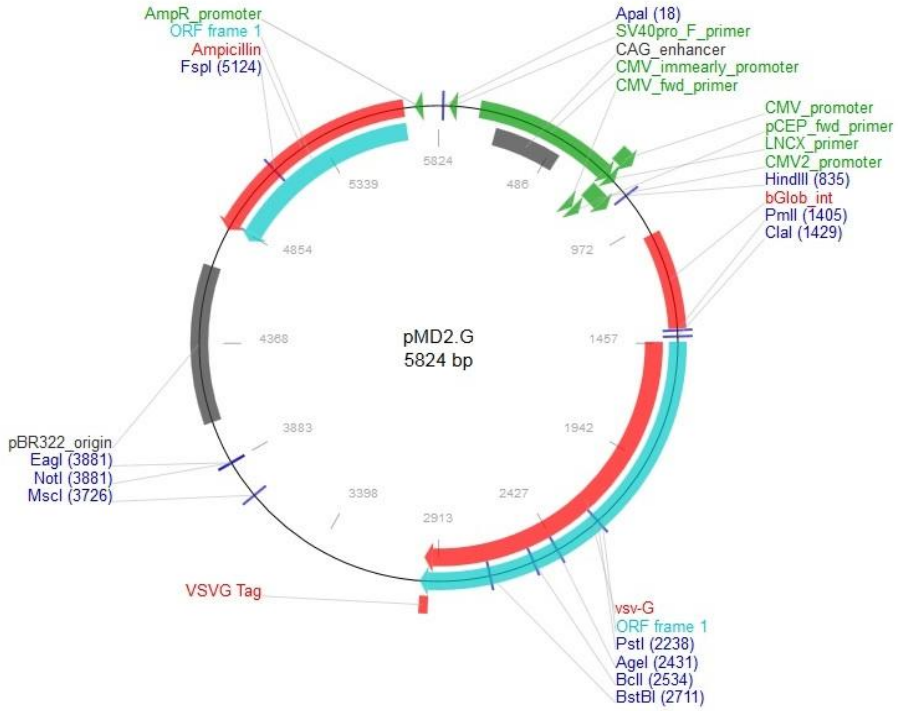



**Figure 6: pMD2.G vector map generated by Addgene from full sequence supplied by depositor.**

#### 2.1.8.2 psPAX2

The plasmid is a second-generation packaging plasmid used to produce human

immunodeficiency virus 1 (HIV-1) derived lentivectors, which encodes for the Gag/Pro/Pol genes derived from HIV-1. The promoter is the chicken beta actin promoter and the polyadenylation signal is the rabbit beta globin polyA. Resistance to Ampicillin. psPAX2 was a gift from Didier Trono at the Swiss Federal Institute of Technology Lausanne (Addgene plasmid # 12260).

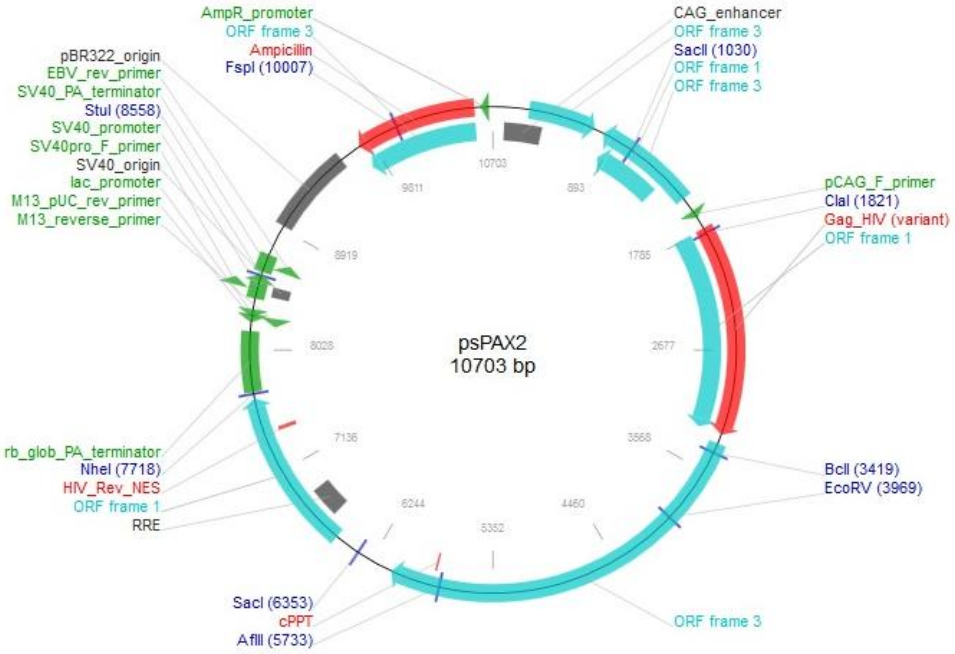



**Figure 7: psPAX2 vector map generated by Addgene from full sequence supplied by depositor.**

### 2.1.8.3 pLVX-Tet-On Advanced

The pLVX-Tet-On Advanced vector constitutively expresses the tetracycline-controlled transactivator, rtTA-Advanced, which is under the control of CMV promoter. In addition to lentiviral elements, this vector contains a geneticin resistance gene to be used for the selection of stable transductants. The vector also contains a pUC origin of replication and an *E. coli* ampicillin resistance gene ($Amp_r$) for propagation and selection in bacteria.

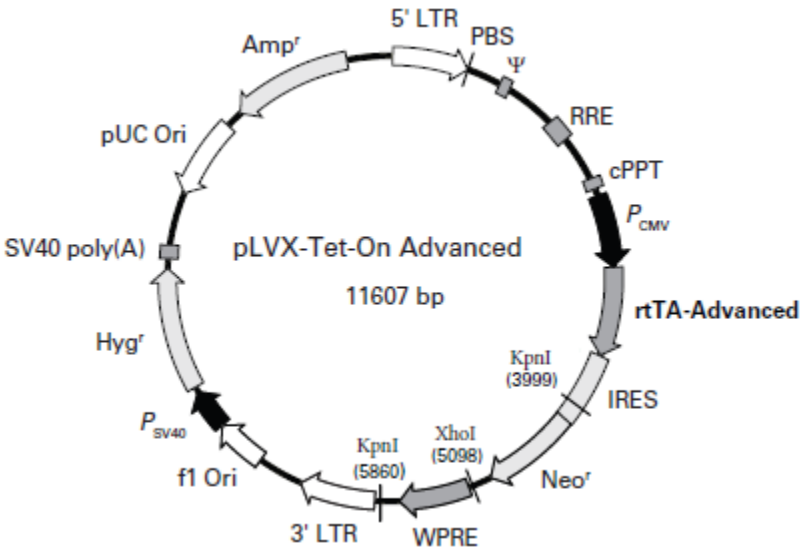



**Figure 8: pLVX-Tet-On Advanced vector map generated by Clontech Laboratories, Inc. (Protocol No.PT3990-5).**

### 2.1.8.4 PLVX-tight-Puro

pLVX-Tight-Puro is a tetracycline (Tet)-inducible, lentiviral expression vector. It is designed to express a gene of interest under the control of *P*Tight, a modified Tet-responsive promoter, which is composed of a modified minimal CMV promoter, and seven direct repeats of a 36 bp regulatory sequence containing the 19 bp Tet operator sequence (*tetO*). This vector is designed to be used with Lenti-X Tet-On Advanced. In addition to lentiviral elements, pLVX-Tight-Puro contains a puromycin resistance gene (*Puro$_r$*) under the control of the murine phosphoglycerate kinase (PGK) promoter (*P*PGK) for the selection of stable transductants. The vector also contains a pUC origin of replication and an *Ampr* for propagation and selection in bacteria. Wild-type PTEN or mutants, wild-type BRAF or BRAF$^{V600E}$ and IGF-1R were cloned into multiple cloning sites between 5'cloning site EcoRI and 3' cloning site BamHI.

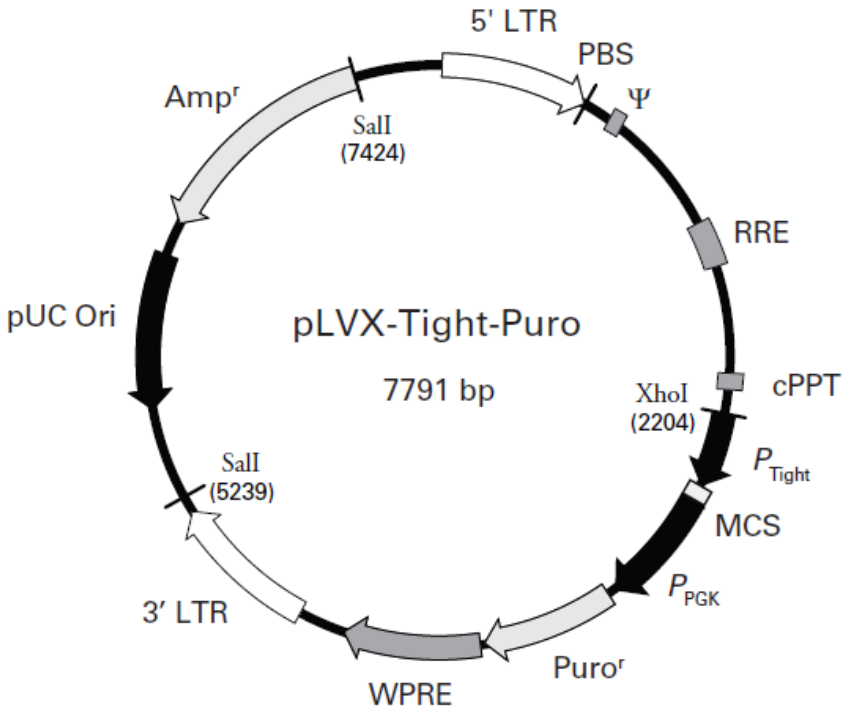



**Figure 9: PLVX-tight-Puro vector map generated by Clontech Laboratories, Inc. (Protocol No.PT3996-5).**

### 2.1.8.5 AKT3 expression plasmid

pcDNA3 are 5.4 kb vectors and designed for high-level stable and transient expression in mammalian hosts. A full length complementary DNA (cDNA) encoding the human AKT3 was subcloned into the mammalian expression vector at the multiple cloning sites between 5'cloning site HindIII and 3' cloning site EcoRI. A c-Src myristoylation sequence and a hemagglutinin (HA)-epitope are fused in frame to the N terminus of the AKT3 coding sequence generating MYR-HA AKT3 which is constitutively expressed under the control of CMV promoter. The myristoylation tethers AKT3 to the inner surface of the plasma membrane so that the AKT3 can be constitutively activated. The pcDNA3 Myr HA Akt3 was a gift from William Sellers at Loyola University Chicago (Addgene plasmid # 9017).

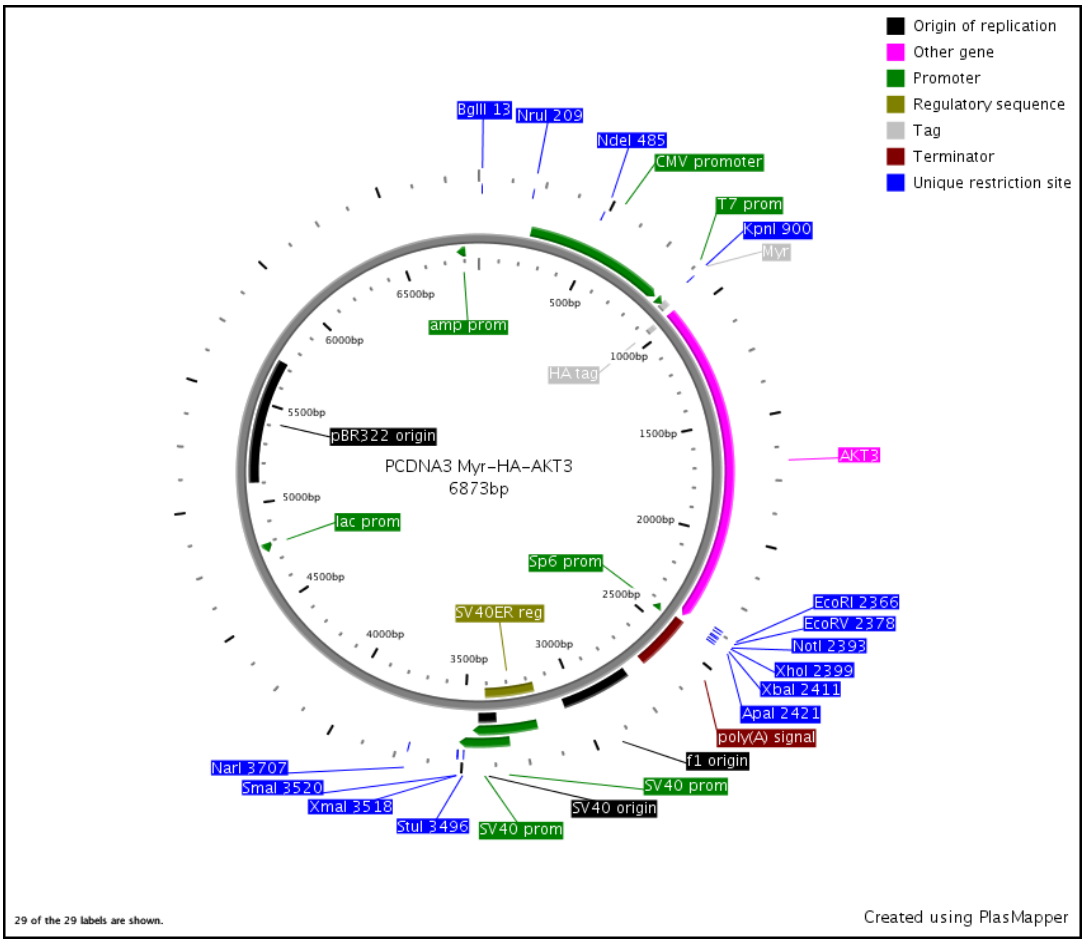



**Figure 10:** **pcDNA3 Myr HA Akt3 vector map created using PlasMapper according plasmid data.**

### 2.1.8.6 pGIPZ lentiviral vector

GIPZ Lentiviral™ Short hairpin RNA (shRNA) was developed by Thermo Scientific™ in collaboration with Dr. Greg Hannon of Cold Spring Harbor Laboratory and Dr. Steve Elledge of Harvard Medical School. shRNA constructs are expressed as human microRNA-30 (miR-30) primary transcripts. A drosha processing site is added to the hairpin construct .The hairpin stem consists of 22 nucleotides (nts) of double-stranded (ds) RNA and a 19 nts loop from human miR-30. Each shRNA construct has been bioinformatically verified to match NCBI sequence data. In addition the vector contains a puromycin resistance gene and TurboGFP gene under the control of the CMV promoter for the selection of stable transductants and the visual marking. Lentiviral shRNA construct against PTEN was gift from Sebastian Haferkamp (Regensburg, Germany).

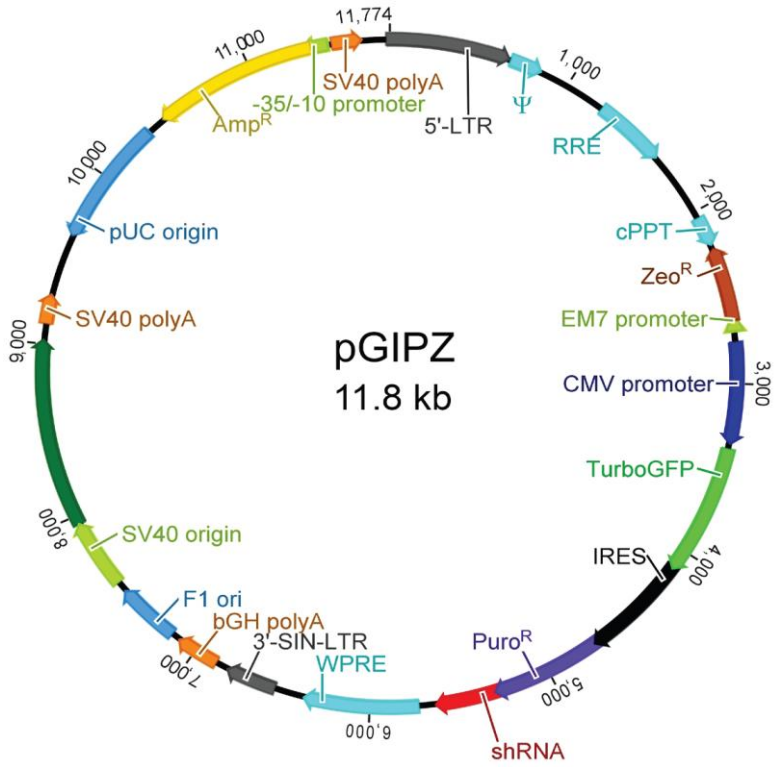



**Figure 11: pGIPZ lentiviral vector map generated by GE Healthcare Dharmacon Inc.** *(http://dharmacon.gelifesciences.com/uploadedFiles/Resources/gipz-lentiviral-shRNA-manual.pdf)*

## 2.2 Cell culture

### 2.2.1 Thawing, passaging and cryopreservation of mammalian cells

Most mammalian cells can be stored in liquid nitrogen for many years. The viability of the cells depends on their ability to cope with the variety of stresses imposed on them during the freezing and thawing procedures.

Cells were taken out of nitrogen storage and thawed rapidly by swirling in the 37°C water bath. 5 ml culture medium is added to cells. After centrifugation, cells are resuspended in the culture medium, pipetted into a T75 culture flask and placed in a $CO_2$ incubator for growth of new culture.

All used human melanoma cell lines were grown in RPMI 1640 medium with 10% FBS, 1% penicillin and streptomycin. For passaging, after washing with PBS, 2 ml trypsin is added. Cells are placed back to the incubator until all of cells were detached from the culture flask. 5 ml culture medium was added to neutralize trypsin. After centrifugation at 1200 revolutions

per minute (rpm) for 3 minutes (Slominski, Tobin et al.) (Megafuge 1.0R), appropriate cell numbers are resuspended in growth medium.

For cryopreservation, cells were trypsinized and pelleted by spinning the cells as gently as possible (1200 rpm, 3 minutes; Megafuge 1.0R), then 1 x $10^6$ cells/ml were resuspended in freezing medium (10% DMSO, 20% FCS, 70% growth medium like RPMI or DMEM). Vials are placed upright in a styrofoam box and covered well, then put in a -20°C refrigerator for 24 hours. Vials were stored upright on ice until placed in a “Mr. Frosty” freezing box containing isopropanol at 4°C. This freezing box is placed at -80°C over night for slow freezing of the cells. Finally, vials are putted in a liquid nitrogen container.

### 2.2.2 Isolation of melanocytes and fibroblasts from human foreskin

Melanocytes and fibroblasts were isolated from human foreskin obtained from circumcision. First subcutaneous fat and blood vessels were removed and the tissue was cut into pieces approximately 5 mm × 5 mm × 5 mm in size. The explants were placed with epidermal side down in 2 ml solution B for about 20 hours at 4 °C for enzymatic digestion. To mechanically separate epidermis from dermis the explants were placed into a petri dish containing 1 ml solution A and scratched repeatedly with a scalpel blade. The isolated epidermal fragments were transferred into a 15 ml tube with 5 ml solution A and melanocytes were dissolved from epidermis by around 60 times resuspending by pipette. After centrifugation at 1200 rpm for 4 minutes (Megafuge 1.0R), the pellets were resuspended in 5 ml melanocyte medium and transferred into a T25 culture flask to grow in a $CO_2$ incubator at 37°C. It could be that fibroblasts and keratinocytes were isolated as well. Due to their higher sensitivity to trypsin melanocytes are able to be separated from the other cells.

Fibroblasts are obtained from the dermis. The dermal explants are placed into one well of a 6 well plate and covered with DMEM medium until fibroblasts grow out. Reaching appropriate confluence fibroblasts were harvested and cultured further.

Table 1: Medium for cell isolation from human foreskin

| Transport medium | | Solution B | | Solution A | |
|---|---|---|---|---|---|
| volume | substrate | concentration | substrate | concentration | substrate |

| | | | | | |
|---|---|---|---|---|---|
| 500 ml | HBSS | 30 mM | Hepes | 30 mM | Hepes |
| | gentamycin | 4 mM | Glucose | 10 mM | Glucose |
| 5 ml | penicillin | 3 mM | KCl | 3 mM | KCl |
| | streptamycin | 130 mM | NaCl | 130 mM | NaCl |
| | amphotericin | 1 mM | Na2HPO4 | 1 mM | Na2HPO4 |
| | | 0,25% | Trypsin | | $H_2O$ |
| | | | $H_2O$ | | |

All of the cell lines used in our study were authenticated by sequence analysis of defined genes. The use and culturing of human skin tissues in this study was approved by the medical ethical committee of the University of Tübingen (43/2008B01; 16/2009B02) and was performed in accordance with the Declaration of Helsinki Principles. All patients provided informed written consent.

### 2.2.3 Plasmid and siRNA Transfektion

In order to deliver plasmids or transfect small interfering RNA (siRNA) into melanoma cells, lipofection is done by using Lipofectamin 2000 or Lipofectamin RNAiMAX. Lipofectamine is able to form cationic liposomes in an aqueous environment, which entrap negatively charged nucleic acid molecules. The nucleic acid containing liposomes with positive charge on their surfaces can fuse with the negatively charged plasma membrane of living cells, which allows nucleic acid to cross into the cytoplasm and contents to be available to the cell for replication or expression.

#### 2.2.3.1 Plasmid transfection

An appropriate number of tumor cells are seeded in a cavity of a 6-well plate, so that the cell density gets 90-95% confluent on the day of transfection. 10 µl lipofectamin 2000 was diluted in 250 µl OPTI-MEM and incubated for 5 minutes, and 4 µg of the plasmid was diluted in 250 µl OPTI-MEM as well, then both of them were mixed very well and incubated 20-30 minutes to form complex of liposomes with plasmid. At the same time the culture medium on cells was replaced by an antibiotic free culture medium at a total volume of 2 ml to avoid cell death during transfection. The 500 µl of complexes were added slowly to cells. After 4 hours the culture medium was exchanged.

#### 2.2.3.2 siRNA transefection

To transfect siRNA to melanoma cells, Lipofectamin RNAiMAX is used, because it has low toxicity and high efficiency to deliver siRNA into cells. For siRNA transfection, cells are plated in a cavity of 6-well plate to get a 50-70% confluent cell density on the day of transfection. siRNA and RNAiMAX are separately diluted in 250 µl OPTI-MEM medium, then mixed together very well, and incubated for 20-30 minutes to form complexes. The culture medium on cells was replaced by antibiotic free medium. The 500 µl of siRNA/RNAiMAX mixture were added to the cells. After 24 hours the culture medium was replaced.

### 2.2.4 Gene delivery by Lentivirus infection

#### 2.2.4.1 $Ca_3(PO_4)_2$-transfektion

$13x10^6$ 293T cells are seeded in T175 culture flask. 3.5 µg of envelope plasmid (pMD2.G), 6.5 µg of packaging plasmid (psPAX2) and 10 µg of expressing plasmids are added individually to 5 ml $H_2O$ in a 15 ml tube, then 500 µl of 2M $CaCL_2$ is added and mixed very well by pipette. 550 µl of 2x BES-buffered solution (Melero, Hervas-Stubbs et al.) are added to the mixture of plasmids in drops while air is blowed to the mixture using an electronic pipette, so that fine crystals can continuously form, which contain the transfected plasmids. The mixture is incubated for 30 minutes at room temperature (RT), then added to cells in drops. After 4 hours the culture medium is refreshed.

| 2X BBS |
| --- |
| 50 mM BES(N,N-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid) |
| 280 mM NaCl |
| 1.5 mM $Na_2HPO_4$ |
| Adjust to pH 6.95 with 1 N NaOH, RT |
| Filter sterilize through a 0.45 µm nitrocellulose filter |

#### 2.2.4.2 Purification of lentivirus

Lentiviral vectors offer unique versatility and robustness as vehicles for gene delivery. They can transduce a wide range of cell types and integrate into the host genome in both dividing

and post-mitotic cells, resulting in long-term expression of the transgene both *in vitro* and *in vivo*.

Lentiviral vectors were produced by cotransfection of a lentiviral transfer and other plasmids for packaging into 293T cells by calcium phosphate transfection method. 2 and 3 days after transfection the supernatants were collected in 50 ml tubes, and centrifuged at 2000 rpm for 10 minutes (Megafuge 1.0R) to pellet any cells/cellular debris. The supernatants were filled into conical tubes and centrifuged for 2 hours at 17000 rpm (ZK401) and 4°C. The supernatants were discarded without disturbing the pellets. Small translucent viral pellets were resuspended in DMEM medium in a total volume of 500 µl. Finally the concentrated viral vectors are transferred to a fresh microcentrifuge tube and 20 µl aliquots were prepared, which are stored at -80°C.

#### 2.2.4.3 Infection of tumor cells with lentivirus

$5x10^5$ cells are seeded in a T25 culture flask. At the next day 50 µl of viral vectors were incubated with 2 µl of polybrene (4 µg/µl) for 5minutes in a sterile 1.5 ml tube. The target cells were brought to room temperature and culture medium was aspirated. 1 ml of fresh culture medium were mixed with viral vectors, transferred completely to the cells and incubated overnight in a humidified incubator in an atmosphere of 5% $CO_2$. At the second day medium containing virus was replaced. After rinsing with PBS, 5 ml of culture medium was added to cells for growing for 48 hours. Puromycin with a proper concentration was added to culture medium until non-transduced cells are completely killed.

### 2.2.5 MUH-Assay

The fluorimetric method using 4-methylumbelliferylheptanoate (MUH) has been developed for detecting cytotoxicity and cell proliferation. The assay is based on the hydrolysis of the fluorochrome by intracellular esterases of viable cells resulting in the production of highly fluorescent 4-methylumbelliferone that can be measured in a microplate fluorimeter.

For the assay 1500 cells/well are seeded in 96-well-plate and incubated overnight. The inhibitors were added to the cells at the indicated concentrations and incubated for 72 hours

at 37°C. After 72 hours the medium on cells was aspirated and the cells were washed with PBS twice. Then 100 µl of the prepared MUH solution (100 µg/ml in PBS) is added to each well and incubation occurred for 1 hour at 37°C in a $CO_2$ incubator. The production of highly fluorescent 4-methylumbelliferone was measured in a microplate fluorimeter (Berthold) with Ex355/Em460 nm.

### 2.2.6 Apoptosis and cell cycle analysis

DNA-binding dyes can bind in proportion to the amount of DNA present in the cell. In this way the cell phases, in which the amount of DNA contents differ, can be divided according to the intensity of the DNA dyes. Due to fragmentation of chromosome during apoptosis the population of apoptotic cells has lower DNA content than that in other phases.

Both floating and attached cells were harvested and fixed in 70% ethanol overnight. Cells were stained with PBS containing 50 µg/ml PI and 50 U RNase A. After 30 minutes cells were measured by LSRII fluorescence-activated cell sorting (FACS; BD, Heidelberg, Germany). The amount of apoptotic cells (sub-G1) was analyzed using the BD FACSDiva software.

### 2.2.7 Two and three-dimensional co-culture

#### 2.2.7.1 Two dimensional co-culture

96 well plates were coated with $1x10^3$ SKMEL19 or dermal fibroblasts, GFP labeled SKMEL19 were seeded onto them and incubated for 24 hours. PLX4032 was added at the indicated concentrations and incubated for another 3 days. After PBS washing, 50 µl lysis puffer (Promega) was added per well. Plates were frozen at -80°C overnight. After thawing and strongly pipetting, 30 µl lysate was added to a black 384 well plate and GFP intensity was measured by a fluorescence microplate reader (Berthold) with Ex485/Em535 nm in triplicates.

#### 2.2.7.2 Three dimensional co-culture (spheroids)

$1x10^3$ SKMEL19 or dermal fibroblasts were seeded in 1% agar noble (Difco) coated 96-well plates for 24 hours. Then GFP labeled SKMEL19 were added and incubated for 2 days. 10

µM PLX4032 was added to the spheroids. Inhibitor was refreshed every 3 days. Cell viability was determined by expression of GFP by fluorescent microscope (Axiovert, Zeiss) up to 8 days.

### 2.2.8 Cell surface and intracellular staining

$5x10^5$ cells were seeded in T25 culture flask and incubated overnight. PLX4032 or AZD6244 was added at indicated concentrations for 24 hours. After PBS washing, cells were detached using 0.2% EDTA/PBS. For surface staining, cell pellets were resuspended in 100 µl cold PBS with IGF-1R antibody or isotype control (BD, Pharmingen) and incubated on ice for 45 minutes. After PBS washing, cells were resuspended in PBS with APC conjugated rabbit anti-mouse antibody (BD, Pharmingen) and incubated in the dark on ice for 30 minutes.

For intracellular staining, cell pellets were fixed in 2% PFA for 10 minutes at RT. After washing with PBS with 2% FBS, cells were resuspended in PBS with 0.5% Saponin containing pERK antibody (Cell Signaling Technology) and 2% FBS for 30 minutes. After washing with PBS with 2% FBS, cells were resuspended in PBS with APC conjugated mouse anti rabbit antibody (Cell Signaling Technology) and 2% FBS for 30 minutes. The intensity of IGF-1R or pERK was measured by LSRII fluorescence-activated cell sorting (FACS; BD, Heidelberg, Germany). Overlay was made by using FCS Express (De Novo software).

### 2.2.9 $IC_{50}$ determination

$2x10^3$ cells were seeded per well into 96 well plates. Picropodophyllin (PPP) was added with gradient concentrations and incubated for 72 hours. Cell viability was measured by MUH assay. We determined the half maximal inhibitory concentration ($IC_{50}$) of PPP, at which 50% of the melanoma cells were killed, using the function of Dose-Response-Inhibition analysis at GraphPad Prism version 5.01 (GraphPad Prism Software Inc.).

### 2.2.10 Immunofluorescence and immunohistochemical analysis

4.5% PFA fixed melanoma cells or sections of paraffin-embedded tumor tissue were blocked, incubated overnight at 4°C with the primary antibodies against IGF-1Rß (Santa Cruz), PTEN

(Cell Signaling), or HMB45 (DAKO), and stained with Cy3- or Cy5-conjugated donkey anti-rabbit or anti-mouse antibodies (Dianova). Nuclei were stained using YO-PRO-1 (Invitrogen). Imaging was performed with a confocal laser microscope (Leica TCS SP).

#### 2.2.11 Stability assay

For the stability assay, the melanoma cells (SKMEL19 and 1205LU) were pretreated with 2 µg/ml doxycycline for the induction of the expression of $BRAF^{V600E}$ or the different PTEN forms (WT, G129E, Y138L, C124S) for 48 hours before cultivation in medium containing 150 µg/ml cycloheximide (CHX) for the indicated time points (0, 3, 6,and 12 hours). To analyze the effect of the MEK inhibitor AZD6244 on protein stability, the cells were instead pretreated for 12 hours with the MEK inhibitor AZD6244 (4 µM). Cells were lysed with lysis buffer (Cell Signaling Technology), and lysates were used to perform Western Blot analyses for the detection of IGF-1R in relation to beta-actin in order to determine the protein stability after inhibiting the protein de novo synthesis using CHX.

## 2.3 Biochemistry

### 2.3.1 Gene transcription

#### 2.3.1.1 RNA Extraction

Total RNA was extracted from cells using NucleoSpin RNA (MACHERY-NAGEL) according to the instruction of the manufacturer.

#### 2.3.1.2 Measurement of the concentration of the isolated RNA/DNA

The absorbance (A) of a diluted RNA sample is measured at 260 and 280 nm. The nucleic acid concentration is calculated using the Lambert-Beer-law, which predicts a linear change in absorbance with concentration. An A260 reading of 1.0 is equivalent to ~40 µg/ml single-stranded RNA. The A260/A280 ratio is used to assess RNA purity. An A260/A280 ratio of 1.8-2.1 is indicative of highly pure RNA. Instead, an A260 reading of 1.0 is equivalent to ~50 µg/ml double-stranded DNA. The A260/A280 ratio of 1.8 is indicative of highly pure DNA.

### 2.3.1.3 Reverse Transcription

Reverse transcriptase can use RNA as template to generate cDNA, which is used for semiquantitative detection of gene expression.

1 µg of RNA was diluted in nuclease free water in a 0.5 ml-PCR-tube at a final volume of 24 µl. To denature RNA templates, the tube was incubated at 70°C for 5minutes, then immediately put on ice for at least 1 minute. At the same time a cDNA synthesis mix was prepared. 16 µl of the mix were added to each tube containing RNA templates. The tubes were mixed gently, and centrifuged briefly, then incubated for 10 minutes at 25°C, followed by 50 minutes at 50°C for 1 hour. Finally the tubes were heated at 85°C for 5 minutes to terminate the reactions and chilled on ice. cDNA synthesis reaction can be stored at −20°C or used for PCR immediately.

Table 2: Reverse transcription

| RNA-Primer-Ansatz | |
|---|---|
| 1 µg | isolated RNA |
| x µl | add DNase/RNase free Water to 24µl |
| Master mix for reverse transcription | |
| 8 µl | 5X buffer |
| 4 µl | DTT |
| 2.5 µl | dNTPs 2 mM of each dNTP |
| 1 µl | random hexamer primer 200ng/µl |
| 0,5 µl | reverse transcriptase 200U/µl |

### 2.3.1.4 Quantitative Real-Time PCR (qRT-PCR)

The real-time, fluorescence-based, quantitative polymerase chain reaction follows the general principle of polymerase chain reaction. The basis of this method is that binding of dye to ds DNA in PCR causes fluorescence of the dye. An increase in DNA product during PCR therefore leads to an increase in fluorescence intensity. Hence DNA concentrations can be quantified by the measuring fluorescence intensity at each cycle. In order to control the differences in RNA isolation and in the efficiency of the reverse transcription reaction from sample to sample and experiment to experiment, the specific gene being studied is quantified

in relation to another gene called a reference gene or housekeeping gene, which is selected for its almost constant rate of expression in both the control and experimental samples.

qRT-PCR was performed with the LightCycler 480 SYBR Green System (Roche, Germany). The relative expression levels of IGF-1R or IGF-1 were determined by calculating the cycle threshold (Ct) values using the ΔΔCt-method with GAPDH or 18S rRNA as a reference gene.

### 2.3.2 Immunoblotting

#### 2.3.2.1 Cell lysate

For lysis of adherent cells, cells were washed once with pre-chilled PBS. Appropriate amount of cold 1x lysis buffer (Cell signaling) was added onto cells. After 5 minutes incubation on ice, cells were scraped down by a cell scraper. The cell lysates were collected in a 1.5 ml tube, and sonicated briefly, then spinned 10 minutes at 14,000 gravity (g) in a cooled microfuge (Biofuge pico). The supernatant was collected for use.

#### 2.3.2.2 Measurement of protein concentration

The Bradford assay, a colorimetric protein assay, is based on an absorbance shift of the dye Coomassie Brilliant Blue G-250. The binding of the dye to the protein stabilizes the blue anionic form and has an absorption spectrum maximum at 595 nm. The increase of absorbance at 595 nm is proportional to the amount of bound dye, and thus to the amount of protein present in the sample.

The Bio-Rad solution was diluted 1:5 with distilled water. 2 µl of samples were added to 198 µl of prepared Bio-Rad solution in a 96-well plate, which was measured using a microplate reader (Berthold) at 595 nm. To calculate the protein concentration of samples a standard curve is produced using a series of protein standards (BSA) diluted in water to final concentrations of 0 (Blank and Mackensen), 156.25, 312.5, 625, 1250 and 2500 µg/ml. 15 – 30 µg of protein are used for immunoblotting.

### 2.3.2.3 SDS-polyacrylamide gel electrophoresis

The binding of SDS to the polypeptide chain of protein imparts an even distribution of negative charge per unit mass, thereby resulting in a fractionation by approximate size during electrophoresis.

A discontinuous polyacrylamide gel is composed of a lower layer (separating gel) for separation of polypeptides by size and an upper layer (stacking gel) including the sample wells, designed to compress protein in samples into micrometer thin layers when they reach the separating gel. The acrylamide concentration of the gel ranges from 5% to 15%. Lower percentage gels are better for resolving very high molecular weight molecules, while much higher percentages are needed to resolve smaller proteins.

According to the size of detected proteins the SDS-gels with different concentration of separating gel are produced. Prior to electrophoresis the protein samples were combined with1x Laemmli buffer and heated at 95°C for 5 minutes, cooled and 15-30 µg protein per lane were loaded onto the prepared gels. Gels were running for 10 minutes at 70 voltages, followed by 90 minutes 120 voltages.

Table 3: Production of SDS-Gels

| | Separating gel 10% (20 ml) | Separating gel 12% (20 ml) | Stacking gel (10 ml) |
|---|---|---|---|
| $H_2O$ | 9,575 ml | 8,6 ml | 7,225 ml |
| 40% Acrylamid | 5,025 ml | 6,0 ml | 1,275 ml |
| 1,5 M Tris (pH 8,8) | 5,0 ml | 5,0 ml | |
| 1,0 M Tris (pH 6,8) | | | 1,25 ml |
| 10% SDS | 200 µl | 200 µl | 100 µl |
| 10% Ammoniumpersulfat (APS) | 200 µl | 200 µl | 100 µl |
| TEMED | 20 µl | 20 µl | 10 µl |

| 6x Laemmli buffer |
|---|
| 10 ml 1M Tris pH 6.8<br>30 ml Glycerin<br>12 g SDS<br>30 µl ß-Mercaptoethanol |

| |
|---|
| 50 µg Bromphenolblau<br>add $H_2O$ to 100 ml |

| 5x Running buffer |
|---|
| 15.1 g Tris(base)<br>94 g Glycin<br>5 g SDS<br>add $H_2O$ to 1 litre |

#### 2.3.2.4 Western Blot

In order to make the proteins accessible to antibody detection, they are transferred from the gel onto a membrane made of nitrocellulose or polyvinylidene difluoride (PVDF). The proteins maintain the organization they had within the gel. Electrophoretic transfer is used due to its high speed and transfer efficiency.

A protein-containing polyacrylamide gel was placed in direct contact with a piece of PVDF and 3 filter papers on top of that, then "sandwiching" this between two electrodes submerged in a conducting solution. An electric field of 25 voltages was applied overnight so that the proteins move out of the polyacrylamide gel and onto the surface of the membrane, where the proteins become tightly attached.

Since the membrane used in Western Blotting have a high affinity for proteins, after the transfer of the proteins from the gel, it is important to block the remaining surface of the membrane to prevent nonspecific binding of the detection antibodies during subsequent steps. The membranes were rinsed briefly with Tris-Buffered Saline with Tween 20 (TBST) buffer, then incubated with 5% non-fat dry milk in TBST buffer for at least 1 hour. Following blocking, the corresponding antibody, which recognizes a specific protein or epitope, was diluted in 5% milk buffer. Membranes were incubated overnight at 4°C with the antibody solution in a 50 ml tube under gentle agitation (RM5, Fröbel Labortechnik). At the next day the membranes were rinsed three times with TBST buffer, 10 minutes for each time. Because the primary antibody, which recognizes the target protein in a Western Blot, is not directly detectable, tagged secondary antibodies are used for detection. The secondary antibodies were also diluted in 5% milk buffer in a new 50 ml tube. Membranes were incubated at RT for 1 hour with the secondary antibody; subsequently membranes were rinsed three times, 5

minutes for each time. Since the secondary antibodies were labeled with Horseradish peroxidase (HRP), chemiluminescent substrates are used. 700 µl of the substrates are added on each membrane, and then the membrane is sealed in plastic. Light signals are documented with X-ray film. Semi-quantification of western blots was done with the freeware software tool scion image (NIH image, http://rsb.info.nih.gov/nih-image/) by using the actin signal for normalization.

| Transfer buffer |
| --- |
| 5.8 g Tris(base) |
| 2.9 g Glycin |
| 0.37 g SDS |
| 200 ml methanol |
| add $H_2O$ to 1 litre |

| TBST |
| --- |
| 2.42 g Tris(base) |
| 8 g NaCl |
| adjust to pH 7.6 with 1 N NaOH, RT |
| add $H_2O$ to 1 litre |
| add 1 ml Tween 20 |

## 2.4 Molecular biology

### 2.4.1 Transformation of competent E.coli with plasmids

Competent E.coli bacteria were thawed on ice for 5 minutes, then 10 ng of plasmids were added. After mixing with pipette the mixture was incubated on ice for 20 minutes so that plasmids can attach to the competent cells. Subsequently the competent cells were heated at 42°C for 90 seconds to create a thermal imbalance on either side of the cell membrane, which forces the DNA to enter the cells through either cell pores or the damaged cell wall. The cells were shortly put on ice again, before 1 ml of lysogeny broth (LB) medium was added to the cells. After growing at 37°C for 1hour, 200 µl of the cell suspension were plated on a LB-agar plate containing 50 µg/ml ampicillin. To obtain a great amount of desired plasmids, transformed bacteria are cultured in LB-medium with appropriate antibiotic overnight at 37°C

under gently agitation.

| LB Medium |
| --- |
| 10 g Trypton |
| 5 g yeast extract |
| 5 g NaCl |
| add $H_2O$ to 1 litre |

| LB-agar plates |
| --- |
| 10 g Trypton |
| 5 g yeast extract |
| 5 g NaCl |
| add 15 g Agar |
| add $H_2O$ to 1 litre and autoclave |
| use around 20 ml per plate |

### 2.4.2 Plasmid preparation

To obtain a great amount of desired plasmids, transformed bacteria were cultured in LB-medium with appropriate antibiotic overnight at 37°C under gently agitation. The suspension of bacteria was centrifuged at 6000 rpm for 5 minutes at 4°C (Varifuge 3.0R). The pellet was resuspended in 4.5 ml of lysis buffer. 10 ml of 0.2 M NaOH with 1% SDS were added and mixed by inverting tubes several times. Under alkaline conditions (pH 12.0-12.5) both chromosomal DNA and protein are denatured, the plasmid DNA however, remains stable. Then, 7.5 ml of 3 M potassium acetate buffer were added to neutralize the pH-value of the solution so that the large and less supercoiled chromosomal DNA and proteins precipitate, but the small bacterial DNA plasmids stay in solution. After incubation on ice for 15 minutes, the mixture was centrifuged for 5 minutes at 6000 rpm and 4°C (Varifuge 3.0R). The supernatant was filtered through a piece of gauze into a new 50 ml tube. To precipitate plasmid, 13.5 ml of isopropanol were added. After incubation for 15 minutes at RT, the solution was centrifuged for 10 minutes at 6000 rpm and 4°C (Varifuge 3.0R). The pellet was briefly dried, then resuspended in 2 ml of TE buffer. To precipitate RNA and small broken

pieces of genomic DNA, 2 ml of 4 M LiCl were added. The solution was mixed and incubated on ice for 5 minutes, then centrifuged for 5 minutes as before. The supernatant was collected in a new 15 ml tube, and 10 ml of pre-chilled 100% ethanol were added to precipitate plasmids. The tube was placed at -20°C overnight. At the next day plasmids were pelleted by centrifugation for 5 minutes at 6000 rpm and 4°C (Varifuge 3.0R). After washing with 70% ethanol and drying briefly, pellet is resuspended in 500 µl of TE buffer. 5 µl of RNase were added to degrade the remaining RNA, which is co-precipitated with plasmids. After incubation for 30 minutes at 37°C, 50 µl of 3 M sodium acetate pH 5.6 were added. To dissolve and denature proteins, 550 µl of phenol/chloroform (phenol:chloroform:isoamylalkohol, 25:24:1) were added. After strongly shaking for 2 minutes, the solution was centrifuged for 3 minutes at 13000 rpm and 4°C (Biofuge pico). As much as possible from the upper water phase which is containing plasmids was collected in a new 1.5 ml tube, and the procedure of purification was repeated again. Then 400 µl of chloroform were added to the collected water phase. After shaking and centrifuging, the upper water phase was transferred into a new 1.5 ml tube. 250 µl of isopropanol were added to precipitate plasmids. After incubation for 5 minutes at RT plasmids were pelleted, washed with 70% ethanol, and dried at 37°C. Then the plasmids were resuspended in an appropriate volume of Tris-EDTA (TE) buffer.

| Lysis buffer |
| --- |
| 50 mM Glucose |
| 25 mM Tris/HCl pH 8 |
| 10 mM EDTA |

| Potassium acetate buffer |
| --- |
| 29.4 g $KCH_3COO$ |
| 5 ml $CH_3COOH$ |
| add $H_2O$ to 100 ml |

| TE buffer |
| --- |
| 10 mM Tris/HCl pH 8 |
| 1 mM EDTA |

### 2.4.3 Sequencing

Sequencing analyzes the order of individual nucleotides present in molecules of DNA or RNA, which has become indispensable for basic biological research, and in numerous applied fields such as medical diagnosis, biotechnology, forensic biology, virology and biological systematics.

In 1977, Frederick Sanger and colleagues developed Didesoxy Sequencing or chain termination, based on the use of didesoxynucleotides in addition to the normal nucleotides found in DNA. Due to lack of a 3'-OH group, integration of these modified nucleotides into a sequence prevents the addition of further nucleotides, because a phosphodiester bond cannot form between the dideoxynucleotide and the next incoming nucleotide, and therefore the DNA chain is terminated. These chain-terminating nucleotides may be radioactively or fluorescently labeled for detection in automated sequencing machines.

BRAF, PTEN and mutants were cloned into inducible lentiviral vectors (PLVX-tight-PURO, Clontech) by using In-fusion-HD Liquid Kits (Clontech) according manufacturer's protocol and Sanger-sequenced for verification (GATC Biotech, Konstanz, Germany).

### 2.4.4 Gel electrophoresis

Due to carrying negatively charged oxygens in the phosphate groups in the DNA backbone DNA molecular has an overall negative charge. In an electric field it moves towards the positive pole of the electrophoresis chamber. As it moves through the pores of the agarose matrix, the smaller one migrates faster and further over a given period of time than do the larger. Therefore DNA molecular can be separated by size.

1 mg of agarose was added to 100 ml of Tris-Borate-EDTA (TBE) buffer. It was heated in microwave until agarose was dissolved completely. 10 µl of Gelred were added to the solution, then it was poured into a 35 ml gel form and combs for slot formation were added. After solidification the prepared 1% gel was merged in TBE buffer, then PCR products were

mixed with loading dye and loaded into the slots of the agarose gel. The gel runs for 1 hour at RT with 120 voltages. Under UV-light (280 nm) the PCR product is visible and was cut out for gel extraction.

| 10 X TBE buffer |
| --- |
| 9 M Tris-Base<br>9 M Boric acid<br>250 mM EDTA |

### 2.4.5 Gel extraction

The PCR products are extracted from agarose gel using Qiagen extraction kit according to the instruction.

### 2.4.6 Restriction digest

Restriction enzymes cut DNA at or near specific recognition nucleotide sequences known as restriction sites. Restriction digest is most commonly used as part of the process of the molecular cloning of DNA fragment into a vector (such as a cloning vector or an expression vector) which contains a multiple cloning site with many restriction sites nearby.

In order to clone desired genes into PLVX-tight-puro vector, vector was digested with a combination of restriction enzymes (BMHI and EcoRI). For digestion the latter were pipetted together. The mixture was incubated at 37°C for 2 hours, and after separation by gel electrophoresis digested vectors or inserts were purified by gel extraction.

| Mixture of digestion |
| --- |
| 1.5 µg PLVX-tight-puro vector Plasmid |
| 2 µl BMHI |
| 1 µl EcoRI |
| 4 µl 10x Tango buffer |
| add $H_2O$ to 20 µl |

### 2.4.7 In fusion cloning

With In-Fusion HD Cloning Plus System (Clontech) any oligo/PCR fragment (20 bp to 15 kb) or multiple fragments can be directionally cloned into any linearized vector in a single reaction. Firstly, single-stranded regions at the ends of the oligo/PCR insert and linearized vector are created by the In-Fusion enzyme. Through base pairing of the complementary regions on the

insert and vector DNA molecules they are spontaneously annealed. Any single-stranded gaps of the annealed DNA are repaired when they are transformed into *Escherichia coli,* therefore resulting in the synthesis of a recombinant vector containing the oligo/PCR insert. This system allows sequence and restriction site-independent cloning. No vector dephosphorylation, blunt-end polishing, or PCR fragment digestion is required.

cDNAs of IGF-1R, BRAF, PTEN and individual mutants are used as template for PCR amplification. Double digested vector and PCR products are separated by gel electrophoresis and purified by gel extraction. In-fusion reaction is done according manufacturer's protocol. Mutated BRAF cDNA sequence was a generous gift from Sebastian Haferkamp (Würzburg, Germany). PTEN and PTEN mutant cDNA sequence were kindly provided by Nick Leslie (Heriot-Watt University, Edinburgh, UK). IGF-1R cDNA sequence was bought from ORIGENE. Wild-type BRAF cDNA sequence was amplified by using Human Multiple Tissue cDNA (MTC) Panel II (Clontech).

Table 4: cloning PCR

| Reagent | Volume |
|---|---|
| CloneAmp HiFi PCR Premix | 12.5 µl |
| Primer 1 | 5 pmol |
| Primer 2 | 5 pmol |
| Template (cDNAs) | 100 ng |
| Sterilized distilled water | up to 25 µl |

PCR Reaction

98°C 10 second
55°C 5 or 15 second
72°C 30–60 second/kb
} 30–35 cycles (3-step PCR)

### 2.4.8 Inducible expression of target gene in melanoma cells

The Tet-On Gene Expression Systems were described by Gossen *et al.* (Gossen, Freundlieb et al. 1995*)*. It allows researchers to regulate gene expression in a quantitative manner.

The Lenti-X Tet-On Advanced Inducible Expression System contains two critical

components: the Regulator Vector: pLVX-Tet-On Advanced and the Response Vector: pLVX-Tight-Puro. The pLVX-Tet-On advanced vector constitutively expresses the tetracycline-controlled transactivator, rtTA-Advanced. At present of doxycycline it binds to the inducible promoter (*P*Tight), which controls expression of your gene of interest, and further activates transcription of the downstream gene.

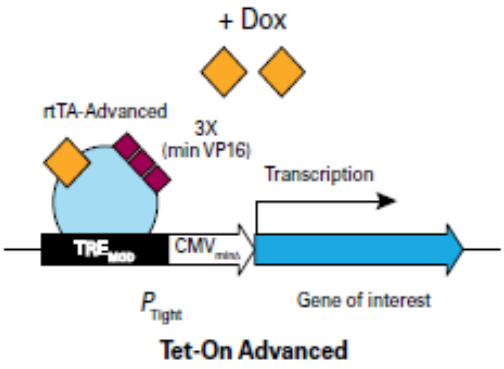



**Figure 12: Gene induction in the Tet-On Advanced System by *Clontechniques*. *Dox: doxycycline**

To establish the complete Tet-On Advanced System, melanoma cells were firstly transduced with Lentiviral vector expressing rtTA-Advanced. The transduced cells were cultured under selection with 200 µg/ml G418 until non-transduced cells were completely killed. Sequently the transduced cells were transduced again with lentiviral vector, pLVX-Tight-Puro containg target genes. The transduced cells were selected again with 10 µg/ml puromycin to kill non-doubly-transduced cells. The established dual transduced cells were used for inducible expression of target gene.

## 2.5 Statistical analysis

Data are presented as mean ± s.e.m (standard error of measurement) for at least three independent experiments. Statistical analysis was performed with a two-tailed unpaired t test, unless otherwise stated. P values of <0.05 were considered to be statistically significant, and P values of <0.01 were considered as highly significant (* presents P-value<0.05; ** presents P-value<0.01; *** presents P-Value< 0.001).

# 3 Results

## 3.1 IGF-1R expression correlates with expression of PTEN in human melanoma cells

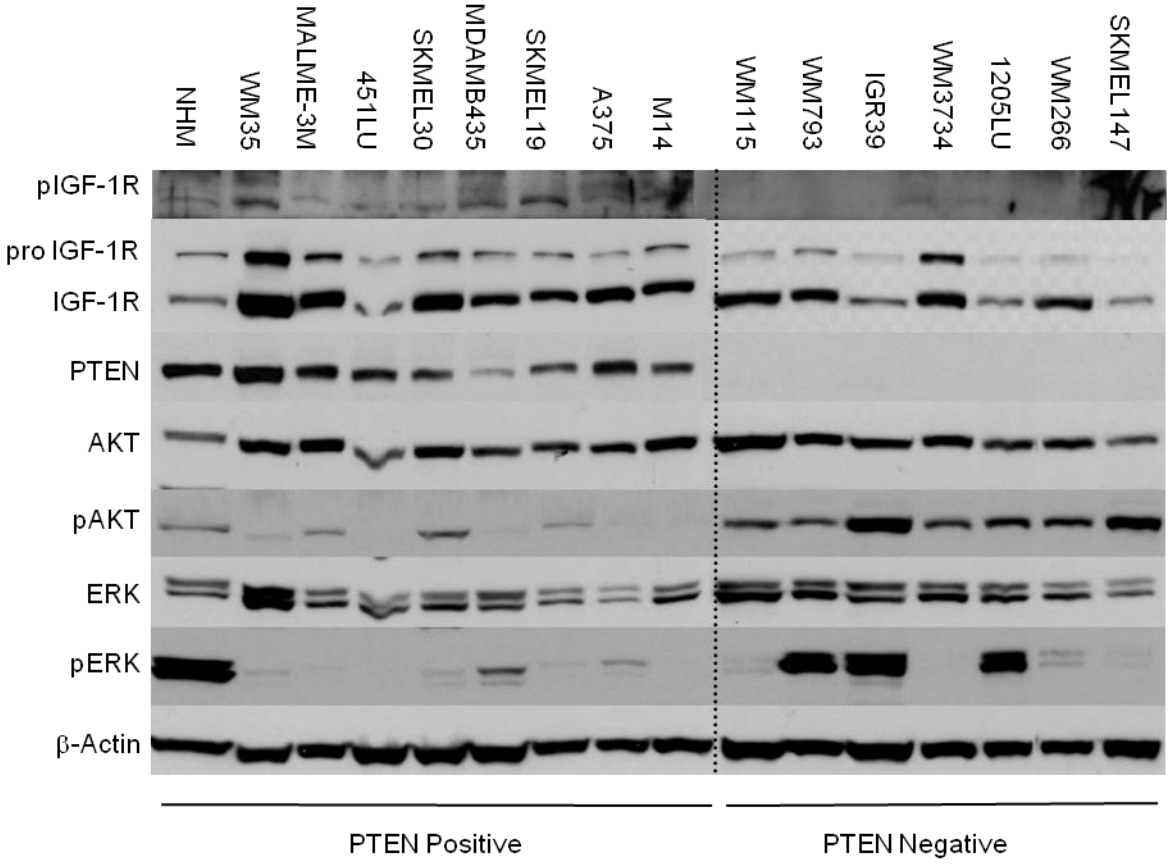
We analyzed expression of IGF-1R in different human melanoma cell lines by qRT-PCR and western blot analyses, and compared it with normal human melanocytes. IGF-1R was significantly upregulated both at the mRNA and the protein level in most of the melanoma cell lines examined compared to normal melanocytes (Figure 13A and B).

Interestingly, we also found that among the different melanoma cell lines the IGF-1R expression level is positively correlated with PTEN expression. PTEN negative melanoma cell lines expressed significantly lower levels of IGF-1R on protein as well as on RNA level than the PTEN positive cell lines (Figure 13A and B).

Due to loss of PTEN, which negatively regulates the PI3K/AKT signaling pathway, the activity of PI3K/AKT signaling as measured by levels of pAKT is significantly higher in PTEN non-expressing melanoma cells than in PTEN expressing melanoma cells (Figure 13A and B). Additionally, we confirmed that there was no correlation between IGF-1R expression and activity of the MAPK signaling pathway, which is mostly mutationally activated in melanoma cells, as measured by pERK levels (Figure 13A).

To determine the level of IGF-1R expression in tissues, we performed semiquantitative immunofluorescence analyses of tissue sections of metastases derived from 35 melanoma patients. The results indicate that in several samples, similar to the expression in melanoma cell lines *in vitro*, IGF-1R expression *in vivo* correlated with PTEN expression in the melanoma tissue samples (Figure 13C). This does not reach significance, probably because only few melanoma cells indeed expressed PTEN at higher levels (Figure 13C).

A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SKMEL30
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SKMEL19
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WM115
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1205LU
WM266
SKMEL147
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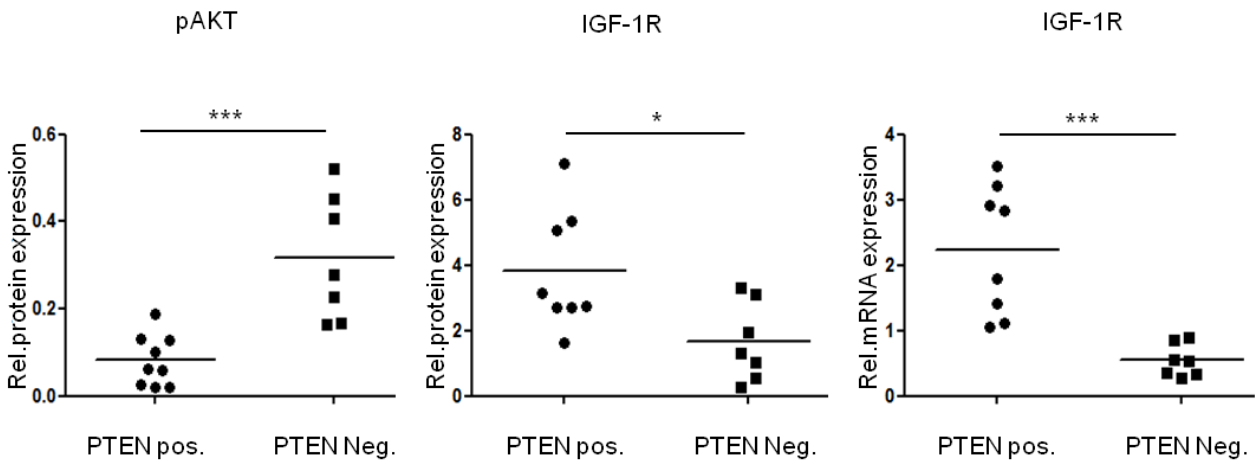
IGF-1R
PTEN
AKT
pAKT
ERK
pERK
β-Actin
PTEN Positive
PTEN Negative

B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IGF-1R
IGF-1R
Rel.protein expression
Rel.protein expression
Rel.mRNA expression
***
*
***
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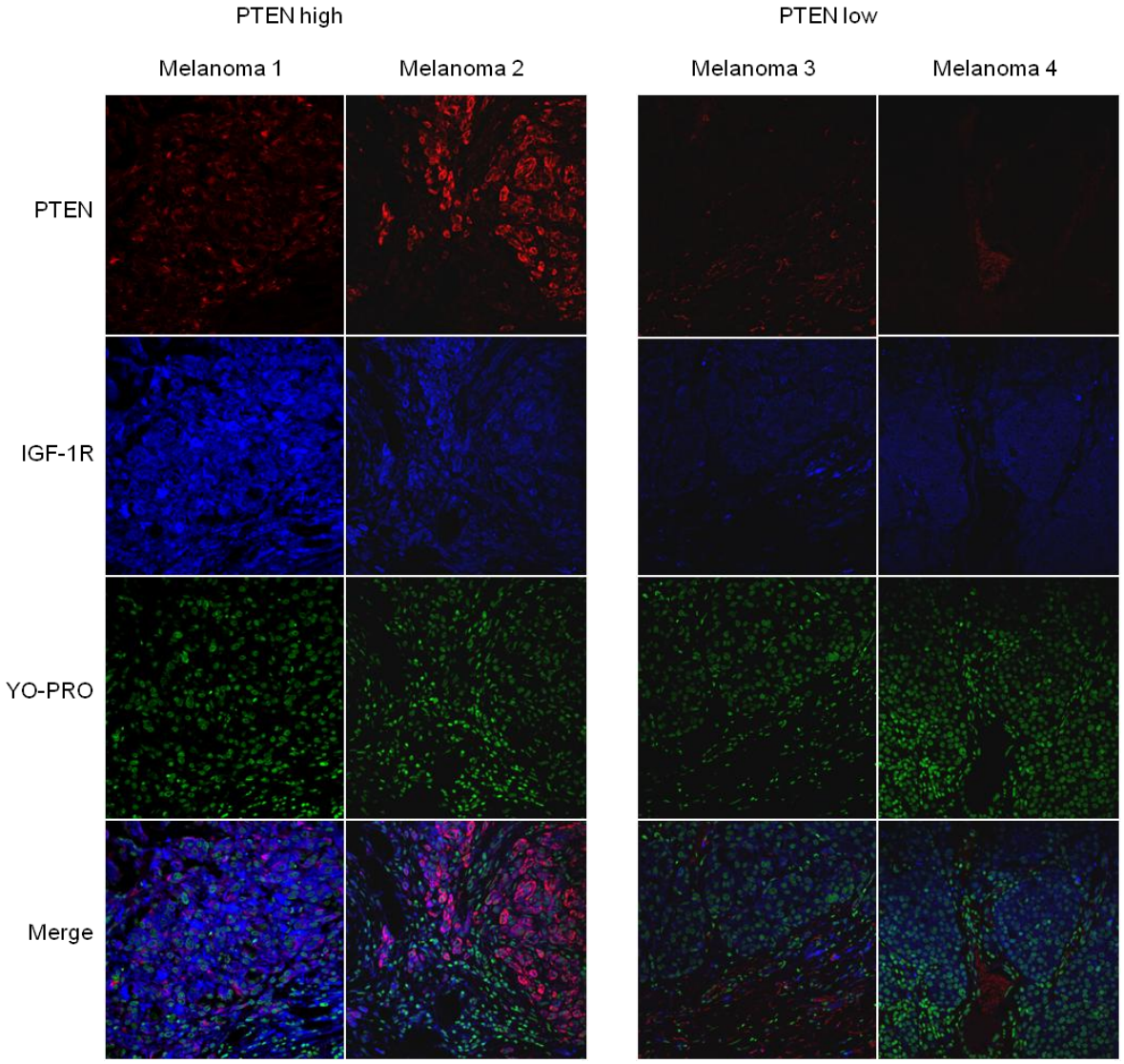
0.4
0.2
0.0
8
6
4
2
0
4
3
2
1
0
PTEN pos.
PTEN Neg.
PTEN pos.
PTEN Neg.
PTEN pos.
PTEN Neg.

C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PTEN low
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Melanoma 2
Melanoma 3
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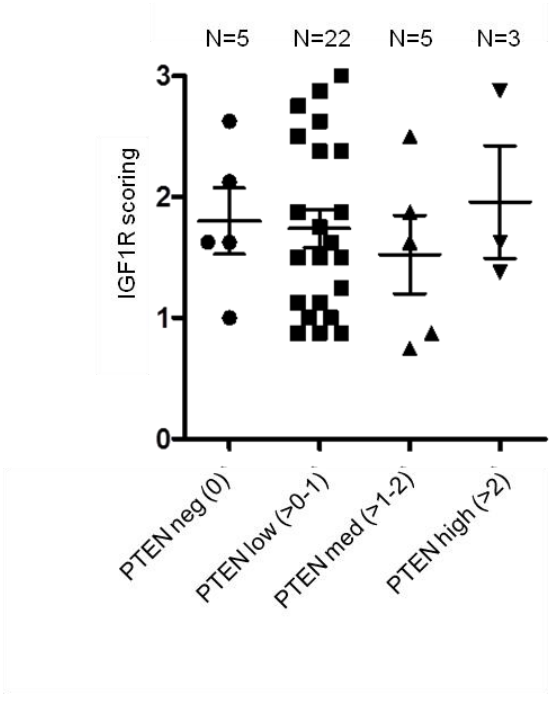
PTEN
IGF-1R
YO-PRO
Merge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N=5
N=3
IGF1R scoring
3
2
1
0
PTEN neg (0)
PTEN low (>0-1)
PTEN med (>1-2)
PTEN high (>2)

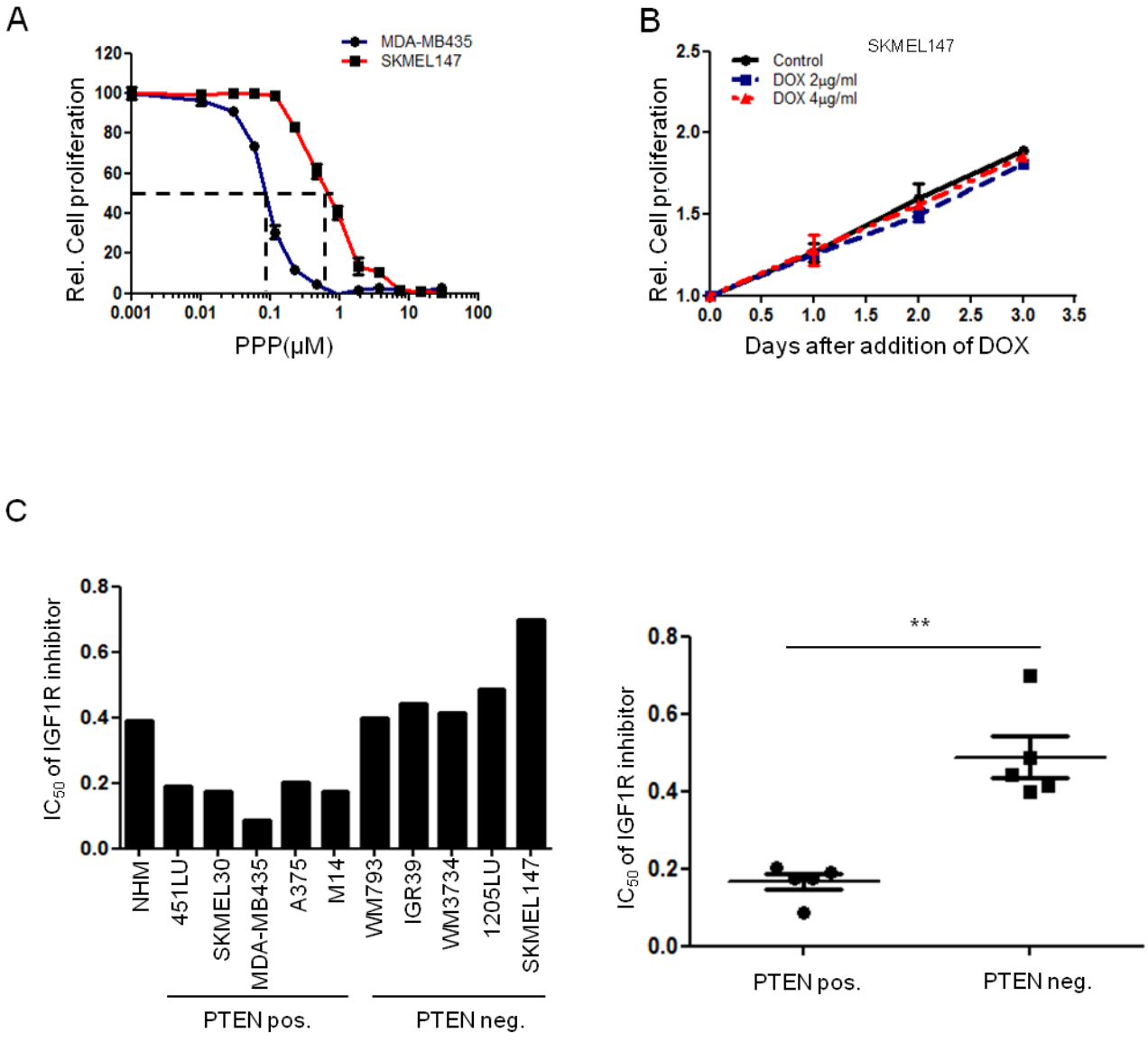
**Figure 13: IGF-1R expression correlates with PTEN expression *in vitro* and *in vivo*.**

(A) 15 different human melanoma cell lines differing in PTEN expression and normal human melanocytes (NHMs) were cultured in RPMI medium for 72 hours. The cell lysates were harvested and immunoblotted with the indicated antibodies. (B) The protein expression of pAKT or IGF-1Rß shown in (A) was normalized to ß-actin (two left panels) or relative RNA expression level of IGF1R determined by qRT-PCR (right panel) in PTEN positive or negative cell lines. RNA expression of IGF-1R in the melanoma cell lines was normalized to the expression level in NHM set as 1. Statistical analysis was done by a Mann Whitney test and stars indicate significant differences. P-value of IGF-1R mRNA expression is 0.0003. P-value of IGF-1R protein expression is 0.0401. P-value of pAKT protein expression is 0.0007. **(C)** Shown is protein expression of PTEN (red) and IGF-1R (blue) in paraffin-embedded human tissue sections of two PTEN high and PTEN low expressing melanoma metastases assessed by representative confocal laser scanning immunofluorescence assay. Nuclei were stained with YOPRO (green). Bottom: 35 biopsies of melanoma metastases were stained as shown in (C), and the level of IGF-1R and PTEN expression were determined using a scoring system ranging from: 0: absent, 1: low expression, 2: medium to 3:high expression. There was no significant correlation between PTEN and IGF-1R expression. For statistical analysis, a Kruskal–Wallis with Dunn's post-test has been done.

## 3.2 PTEN increases the sensitivity of melanoma cells towards IGF-1R inhibition

IGF-1R plays an important role in tumorigenesis, resistance to apoptosis and resistance to anti-cancer agents (Tao, Pinzi et al. 2007; Casa, Dearth et al. 2008; Pollak 2008). IGF-1R has gained increasing attention as a promising target in cancer therapy, but its role as a therapeutic target in melanoma has not been systematically explored yet. We used an allosteric inhibitor of IGF-1R, PPP in a panel of melanoma cell lines with PTEN expression or not and determined their $IC_{50}$, at which 50% of cell growth is inhibited, using a cell growth assay using 4-methylumbelliferrone as a substrate (Figure 14A). Unlike ATP competitive inhibitors, PPP exhibits little effect towards IR (Vasilcanu, Girnita et al. 2004). It has been shown that in uveal melanoma cells with upregulation of the IGF-1R signaling pathway, PPP causes tumor regression and attenuates mechanisms involved in invasion (Girnita, Girnita et al. 2004; Girnita, All-Ericsson et al. 2008). However, cutaneous melanoma cell lines have a different sensitivity to the IGF-1R inhibitor. PTEN positive melanoma cells are significantly more sensitive to IGF-1R inhibition as the PTEN-negative melanoma cell lines (Figure 14C). PTEN-expressing normal human melanocytes (NHMs) are as sensitive as the PTEN-negative human melanoma cell lines (Figure 14C). PPP induces an activation loop-specific inhibition of tyrosine phosphorylation of the IGF-1R (Vasilcanu, Girnita et al. 2004). Thus, the auto-phosphorylation of IGF-1R is suppressed by the PPP in all cells tested,

no matter how their sensitivity towards PPP is (Figure 14D). Surprisingly, expression of IGF-1R is dramatically decreased by PPP only in melanoma cells, which lost PTEN expression (Figure 14D). Interestingly, re-expression of PTEN in the PTEN negative melanoma cell line SKMEL147 led to stabilization of IGF-1R expression after PPP treatment and increased sensitivity towards IGF-1R inhibition (Figure 14E). PTEN re-expression did not affect proliferation of the melanoma cells (Figure 14B). These data suggested that PTEN negative melanoma cells escape IGF-1R blockade by further down-regulation of the receptor and if PTEN is present it stabilizes IGF-1R expression and thereby increases sensitivity specifically towards IGF-1R blockade.



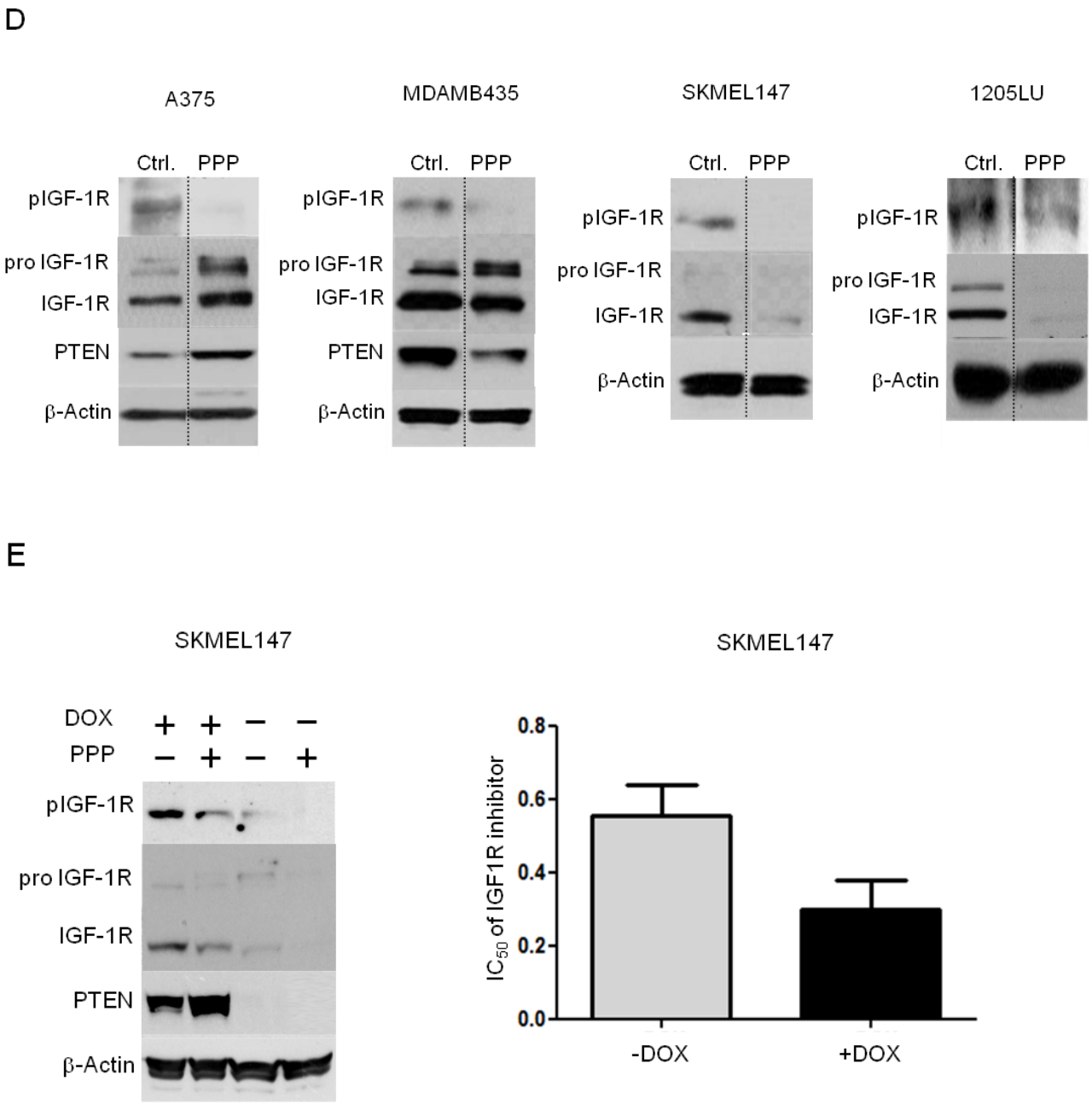



**Figure 14: PTEN expression sensitizes melanoma cells towards IGF-1R inhibition.**

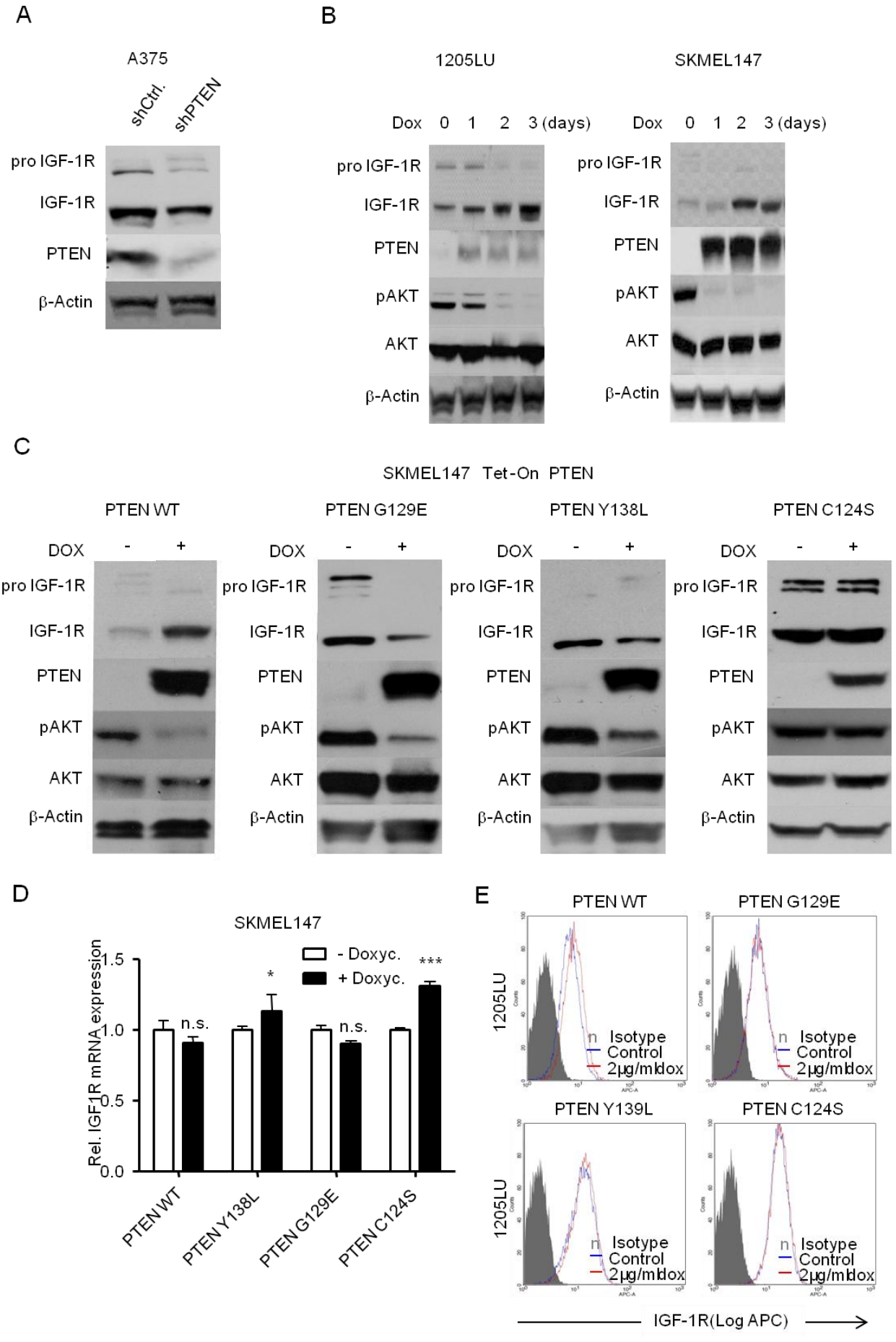
(A) The cell viability of MDA-MB435 (PTEN positive) and SKMel147 (PTEN negative) was assessed by MUH assay after 72 hours of treatment with PPP (0.01-32 µM). The IC50 of PPP was calculated by GraphPad analyse as shown here. (B) The relative proliferation of SKMel147 melanoma cells, in which PTEN expression was induced by treatment with either 2 or 4 µg/ml doxycycline, was assessed by MUH assay. PTEN induction didn´t dramatically influence cell proliferation. **(C)** The cell viability of PTEN positive and PTEN negative human melanoma cell lines and NHM was assessed by MUH assay after 72 hours of treatment with PPP (0.01-32 µM). The results are expressed as $IC_{50}$ of PPP, as shown at the left panel. For statistical analysis, a Mann Whitney test has been done. P-value is 0.0079. **(D)** Cells were treated with 1µM PPP for 24 hours, and the cell lysates were immunoblotted with the indicated antibodies. **(E)** In order to induce PTEN expression in SKMEL147, the cells were treated with 4 µg/ml doxycycline (+) or not (-) as control for 2 days. After that, the cells were treated

with 1µM PPP (+) or not (-) for 24 hours, and the cell lysates were immunoblotted with the indicated antibodies, as shown at the left panel. The right panel showed the mean $IC_{50}$ value from triplicate experiments. For calculating $IC_{50}$, the cell viability of SKMel147 melanoma cells, in which PTEN was either induced (+) by adding 4 µg/ml doxycycline or not (-) for 2 days, was assessed by MUH assay after 72 hours of treatment with PPP (0.01-32 µM). For statistical analysis, a Mann Whitney test has been done. P-value is 0.0204.

### 3.3 The phosphatase activity of PTEN increases IGF-1R protein expression in melanoma cells

To clarify if PTEN regulates IGF-1R expression, we transduced A375 cells via lentiviral gene transfer to express a specific shRNA against PTEN and analyzed changes in the expression of IGF-1R. We found that reduction of PTEN protein levels reduced IGF-1R expression (Figure 15A). In contrast, re-expression of PTEN, using a doxycycline-inducible lentiviral gene transfer, in the PTEN negative human melanoma cell lines SKMEL147 and 1205LU results in a lag increase of IGF-1R expression and in accordance with the role of PTEN, negatively regulating PI3K/AKT pathway, PTEN expression also decreases the level of pAKT (Figure 15B). Our data indicates that IGF-1R expression is directly regulated by PTEN. Further we checked the mechanism by which PTEN regulated IGF-1R expression. As PTEN has dual phosphatase activity, we used 3 PTEN mutants, designed by Nick Leslie, the $PTEN^{G129E}$ mutant lacking the lipid phosphatase activity, but which retains the protein phosphatase activity of the enzyme (Lackey, Barnett et al. 2007), the $PTEN^{Y138L}$ lacking the protein phosphatase activity, but retaining the lipid phosphatase activity (Davidson, Maccario et al. 2010), and $PTEN^{C124S}$, which lacks both types of phosphatase activities (Davidson, Maccario et al. 2010), and established inducible expression of these mutants and wild-type PTEN in SKMEL147 cells. Interestingly, we found that expression of wild-type PTEN induces IGF-1R expression as shown before, but neither of single or both phosphatase-deficient mutants were able to significantly increase expression of the IGF-1R, even single phosphatase-deficient PTEN species decreased its expression (Figure 15C). The similar effect was seen in 1205LU cells overexpressing wild-type or mutant PTEN forms. IGF-1R expression was not comparably induced with mutant PTEN expression as much as with expression of the wild-type PTEN form (Figures 15F). Further we checked the effect of PTEN expression on the transcription level of IGF-1R. Also on transcript level we do see either no or

only an up to two-fold increase in IGF-1R mRNA expression after overexpression of wild-type PTEN (Figure 15D and 15G). This is not enough to explain the more than 7-fold higher protein expression of the IGF-1R (Figures 15C and 15F). In addition, the expression of wild-type PTEN, but not of the mutant forms, increased surface expression of the IGF-1R (Figure 15E). From these results, we postulated that PTEN is able to increase IGF-1R expression in melanoma cells by increasing protein stability of the IGF-1R and this is dependent on the phosphatase activity of PTEN. To address this point, we inhibited protein de novo synthesis with addition of CHX, and analyzed the stability of the IGF-1R in 1205LU melanoma cells, in which wild-type or mutant PTEN had been inducibly re-expressed for 2 days. We analyzed the expression of IGF-1R by Western blot analyses in a time course after CHX treatment. Unlike the expression of the pro-IGF-1R form, expression of IGF-1R was very stable, and does not change significantly during the CHX treatment irrespective of PTEN expression (Figures 15H). Therefore, our results indicate that the stability of IGF- 1R is not significantly different after wild-type or mutant PTEN overexpression (Figures 15H). We conclude that the reason for the higher expression of IGF-1R after wild-type PTEN expression is not a stabilization of the IGF-1R protein and might be achieved by a combination of enhanced IGF-1R transcription and increased protein shuttling, and the phosphatase activity of PTEN seems to be required for this.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pro IGF-1R
IGF-1R
PTEN
β-Actin
B
1205LU
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pro IGF-1R
IGF-1R
PTEN
pAKT
AKT
β-Actin
C
SKMEL147 Tet-On PTEN
PTEN WT
PTEN G129E
PTEN Y138L
PTEN C124S
DOX - +
D
SKMEL147
- Doxyc.
+ Doxyc.
Rel. IGF1R mRNA expression
n.s.
*
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IGF-1R(Log APC)

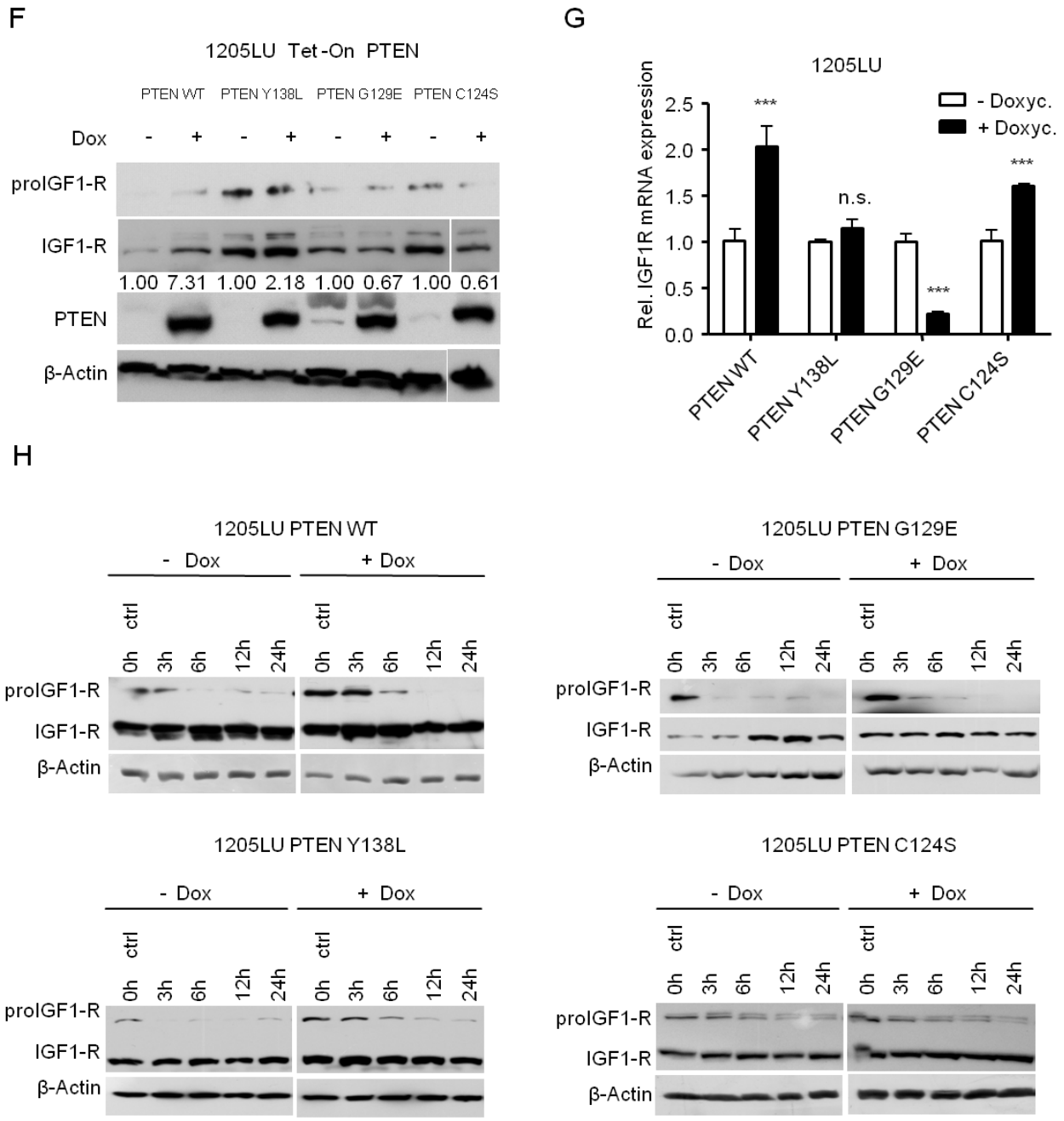



**Figure 15: The phosphatase activity of PTEN increases IGF-1R protein expression.**

**(A)** Control shRNA or shRNA against PTEN was transduced into A375 melanoma cells with a lentivirus expressing system. After 72 hours of the transduction, transduced cells, which co-expressed GFP, were sorted and cultured. After additional 48 hours, the cell lysates were harvested and immunoblotted with the indicated antibodies. **(B)** PTEN was induced to express in 1205LU and SKMEL147 melanoma cells by adding 4 µg/ml doxycycline at the indicated time, and then the cell lysates were harvested and immunoblotted with the indicated antibodies. **(C, F)** wild-type PTEN or the mutants $PTEN^{G129E}$, $PTEN^{Y138L}$ or $PTEN^{C124S}$ lacking defined phosphatase activities was induced to express in SKMel147 (C) and 1205LU (F) melanoma cells. After 48 hours of induction, the cell lysates were harvested and immunoblotted with the indicated antibodies. The protein expression of IGF-1R shown in (F) is normalized to ß-actin, and then the ratio to individual control was determined. **(D, G)** The relative RNA expression level of IGF1R was determined by qRT-PCR. The different

forms of PTEN were induced to express in SKMel147 (D) and 1205LU (G) melanoma cells for 48 hours, and then RNA was prepared for qRT-PCR to detect IGF-1R and 18S rRNA expression. Shown is the relative RNA expression of IGF-1R before (-) or after (+) PTEN induction by adding doxycycline (4 µg/ml). NS: non-significant. **(E)** The surface expression of IGF-1R in 1205Lu melanoma cells after 48 hours of induction of expression of wild-type or mutant PTEN forms was assessed by flow-cytometric analyses. Filled grey field denotes the isotype control staining. **(H)** The stability of IGF-1R and pro-IGF1R was assessed in 1205LU melanoma cells after induction of expression of wild-type or mutant PTEN. After 48 hours of the induction of PTEN expression, cells were cultured in medium containing 150 µg/ml CHX to inhibit the protein de novo synthesis for the indicated time points. Cell lysates were harvested and immunoblotted with the indicated antibodies to detect IGF-1R and its pro-form in relation to beta-actin.

* The experiments of E, F-H have been done by Heike Niessner and Tobias Sinnberg.

### 3.4 The regulation of IGF-1R expression is independent on PI3K and AKT activity in melanoma cells

It has been described that IGF-1R expression can be regulated in mouse embryonic fibroblasts or in a glioblastoma cell line by a negative feedback mechanism of activated PI3K/AKT pathway (Lackey, Barnett et al. 2007; Qin, Li et al. 2009; Chandarlapaty, Sawai et al. 2011). To confirm that, we next investigated the effects of modulation of PI3K/AKT pathway activity on IGF-1R expression. Firstly, we examined the effect of PI3K/AKT inhibition using the inhibitor PI103, BKM120, and GDC0941 and the AKT inhibitor ipatasertib (AKTi) in the PTEN positive melanoma cell lines A375 and MDAMB435 and the PTEN negative melanoma cell lines SKMEL147 and 1205LU on IGF-1R expression by western blot analyses. Treatment with PI3K inhibitors efficiently reduced PI3K/AKT signaling activity in all cell lines already 1 day after treatment as measured by pAKT levels, whereas the AKT inhibitor increased pAKT levels presumably by a positive feedback mechanism (Figure 16A). None of the treatments resulted in elevated expression of IGF-1R in melanoma cell lines (Figure 16A). Consistent with our results, knockdown of AKT3, the dominant isoform of AKT, in the PTEN expressing melanoma cell line A375 or the non expressing cells 1205LU and SKMEL147, using specific siRNA, had an inhibitory effect on AKT phosphorylation, but didn’t increase IGF-1R expression (Figure 16B). Inversely, overexpression of the AKT3 in melanoma cells A375, 1205LU, or SKMEL147 led to increased phosphorylation of AKT, but had no significant effect on IGF-1R expression (Figure 16B). Together these data suggest that in contrast to studies with mouse embryonic fibroblasts and glioblastoma cells (Lackey, Barnett et al. 2007;

Qin, Li et al. 2009; Chandarlapaty, Sawai et al. 2011), IGF-1R expression is independent on the activity of PI3K/AKT pathway in melanoma cells.

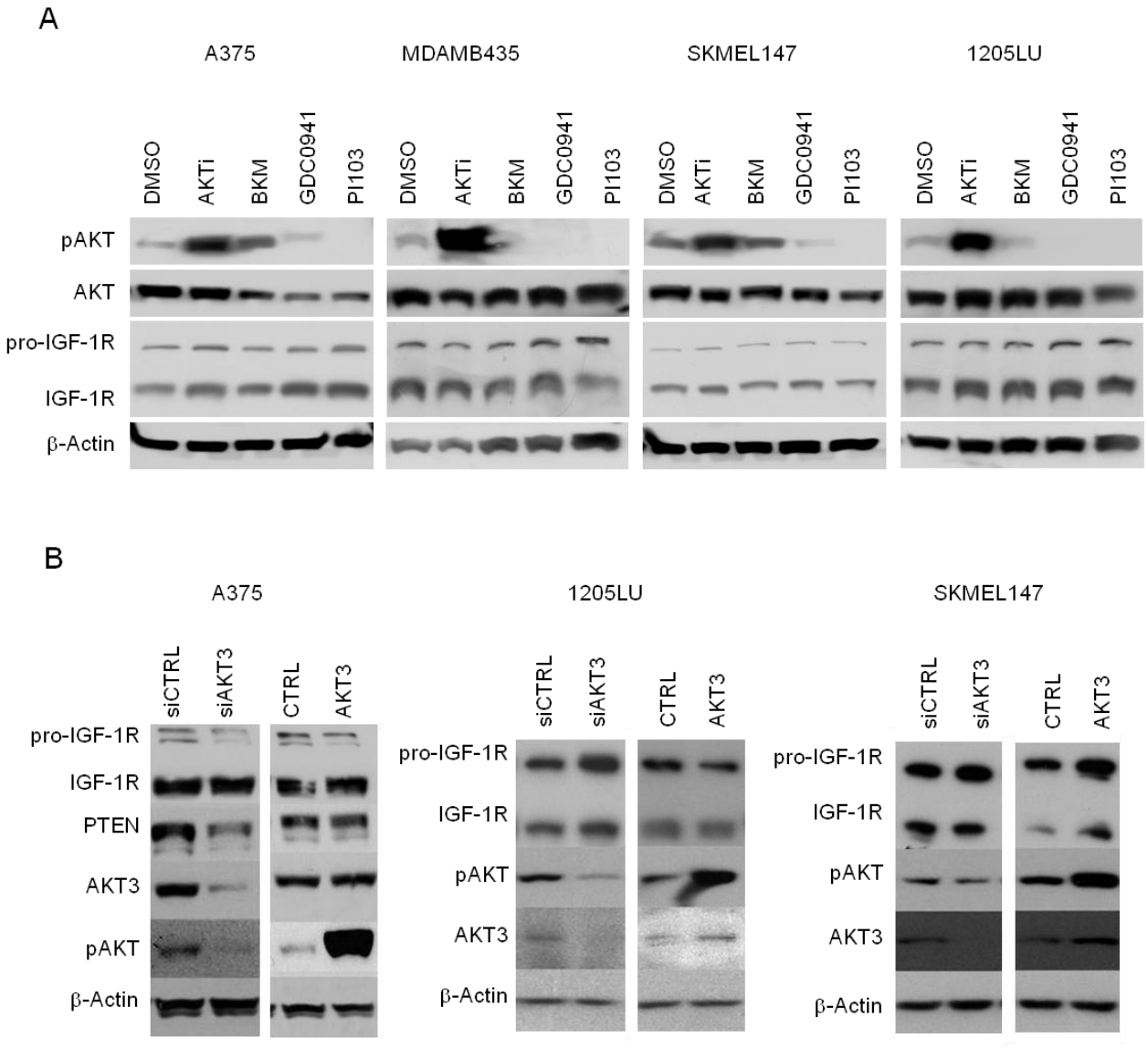



**Figure 16: IGF-1R expression doesn't greatly depend on the activity of the PI3K/AKT signalling pathway in melanoma cells.**

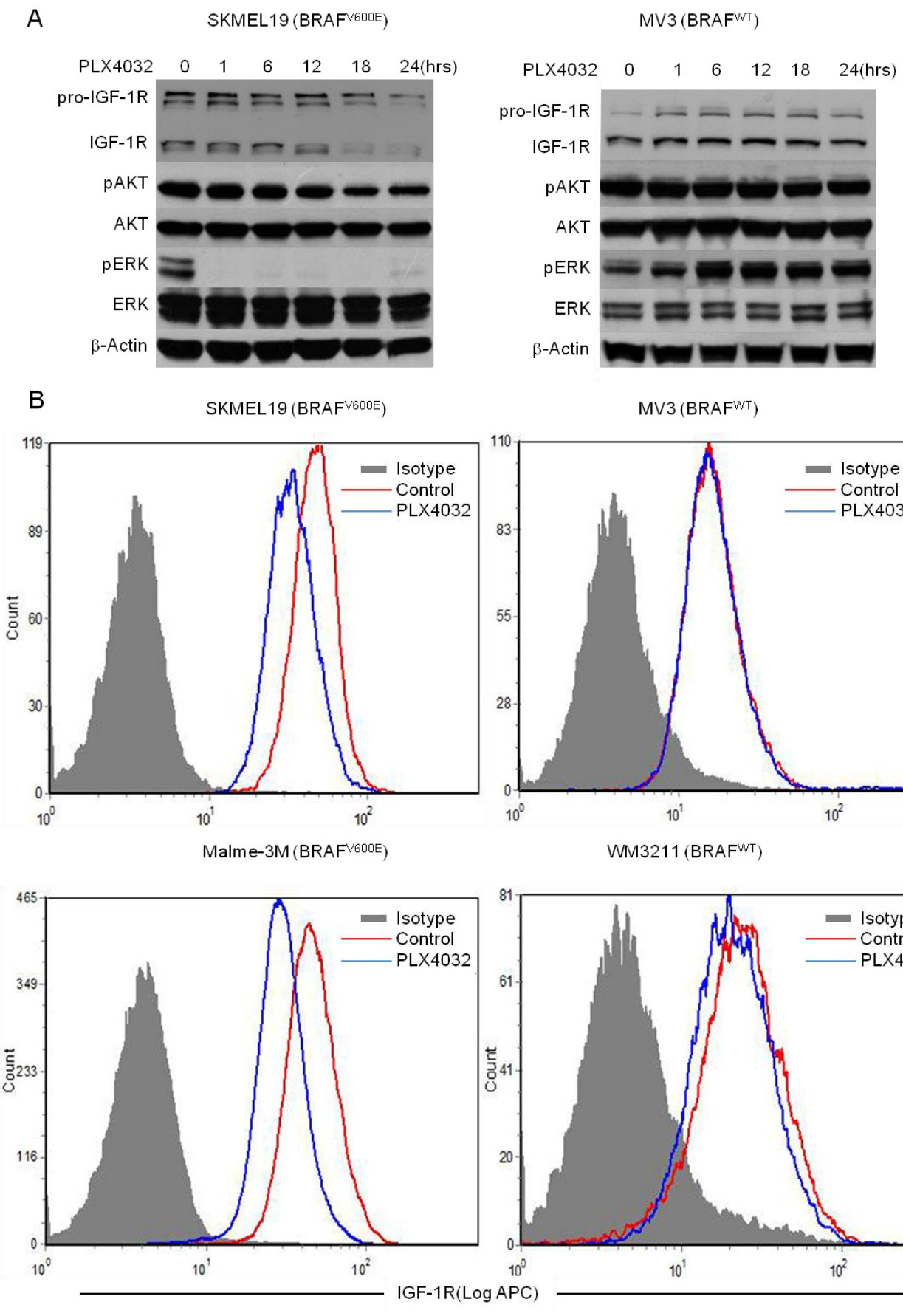
**(A**) The PTEN positive melanoma cell lines A375 and MDAMB435 and the PTEN negative melanoma cell lines 1205LU and SKMEL147 were treated with AKT inhibitor ipatasertib (AKTi: 0.5 µM), the class I pan-PI3K inhibitor buparlisib (BKM120: 1 µM), the class I PI3K inhibitor pictilisib 24(GDC0941: 1 µM) and the PI3-Kinase inhibitor PI103 (2.5 µM) or DMSO for 24 hours and the cell lysates were immunoblotted with the indicated antibodies. **(B)** Scrambled siRNA or siRNA against AKT3 for downregulation of AKT3 expression (left blot) or a control vector or Myr-HA-AKT3 plasmid for overexpression of AKT3 (right blots) was transfected into the PTEN positive melanoma cell lines A375 and the PTEN negative melanoma cell lines 1205LU and SKMEL147. After 48 hours

of transfection, the cell lysates were harvested and immunoblotted with the indicated antibodies.
* Heike Niessner has done the experiments of A.

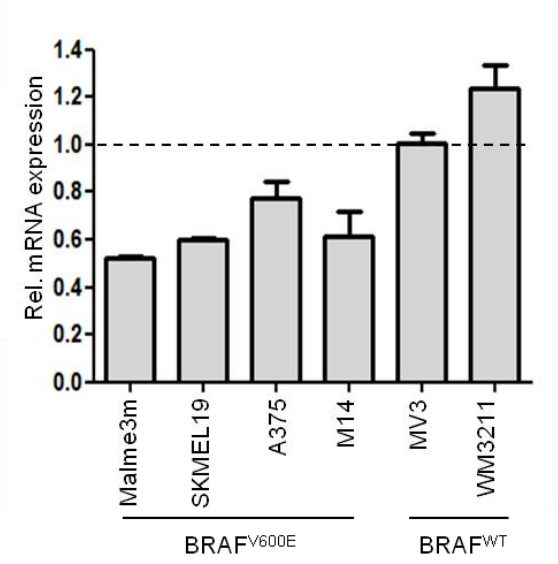
### 3.5 BRAF enhances IGF-1R expression independent on MAPK signaling activity

Due to an activating $BRAF^{V600E}$ mutation, MAPK signaling is constitutively activated in about half of all melanoma patients (Davies, Bignell et al. 2002). Targeting $BRAF^{V600E}$ with the BRAF inhibitor PLX4032, which inhibits specifically $BRAF^{V600E}$, leads to tumor shrinkage and PFS of 7 months in patients with $BRAF^{V600E}$ mutant melanomas (Flaherty, Puzanov et al. 2010). A publication showed that after chronic treatment with PLX4032, melanoma cells could acquire resistance to the BRAF inhibitor, mediated by upregulation of IGF-1R/PI3K signaling (Villanueva, Vultur et al. 2010). On the contrary, another publication showed that stimulation with IGF-1, the cognate ligand for IGF-1R, cannot confer initial resistance against PLX4032 treatment to melanoma cells (Straussman, Morikawa et al. 2012). It seems that IGF-1R signaling has a complex role in the treatment with PLX4032. Actually, it is also possible that IGF-1R expression is regulated differently by targeting BRAF upon short-term exposure to PLX4032. To confirm this hypothesis, we treated the human melanoma cell line SKMEL19, harboring a $BRAF^{V600E}$ mutation, with the inhibitor PLX4032. To preclude the off-target effect, we also used MV3, which has wild-type BRAF. The result showed that in SKMEL19 PLX4032 was effective in reducing pERK levels already 1 hour after treatment (Figure 17A). Interestingly, we also found that the IGF-1R protein expression was decreased, starting at 18 hours after addition of PLX4032, accompanied by a reduction of pAKT levels (Figure 17A), whereas PLX4032 did not affect IGF-1R expression and pERK levels in the melanoma cell line MV3 (Figure 17A). In addition, surface expression of the IGF-1R, where it comes in contact with its cognate ligand and mediates downstream signaling transduction, was also reduced after PLX4032 treatment in SKMEL19, but not MV3 (Figure 17B). This effect on IGF-1R surface expression could be reproduced in two other melanoma cell lines carrying either mutated or wild-type BRAF, respectively (Figure 17B bottom). PLX4032 treatment resulted in down-regulation of IGF-1R expression already on transcriptional level and was evident in several BRAF-mutated melanoma cell lines, but not in those carrying wild-type BRAF (Figure 17C). These data indicate that unlike in resistant melanoma cells, not

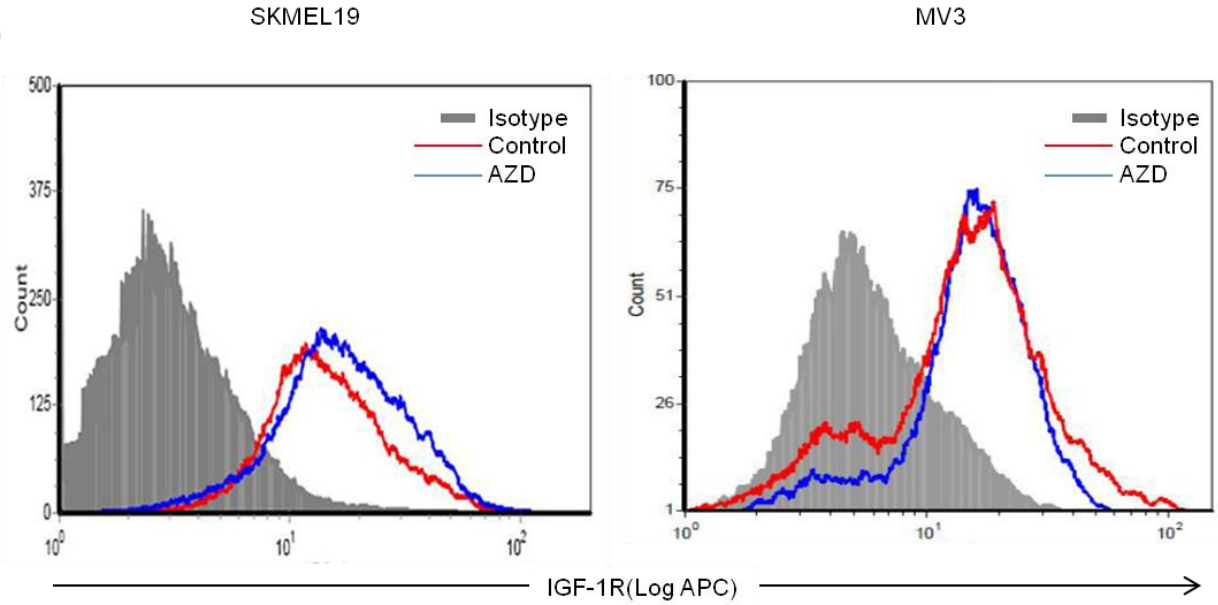
only the total expression of IGF-1R but also the localization on the cell membrane were strongly decreased in melanoma cells after initial treatment with PLX4032. Further we asked whether the decreased expression of IGF-1R is dependent on the activity of the MAPK pathway or not. We inhibited MAPK-signaling using AZD6244, an inhibitor of MEK, which is a downstream component of BRAF in the MAPK pathway. Interestingly, we found that neither IGF-1R surface expression nor total IGF-1R protein expression in the melanoma cells tested in this assay, were decreased by inhibition of MAPK pathway by AZD6244 (Figure 17E and 17D). These data suggest that IGF-1R expression and activity is regulated by BRAF, which is independent on MAPK signaling activity. To proof this we down-regulated BRAF in SKMEL19 or MV3 melanoma cells lines carrying either wild-type or mutated $BRAF^{V600E}$, respectively. In both cell lines down-regulation of BRAF decreases IGF-1R expression (Figure 17F). In addition, up-regulation of either $BRAF^{V600E}$ or wild-type BRAF using a doxycycline-inducible system in SKMEL19 melanoma cells increased IGF-1R expression already 1 day after BRAF induction (Figure 17F). Moreover, $BRAF^{V600E}$ overexpression in SKMEL19 melanoma cells resulted in higher IGF-1R surface expression (Figure 17G). The RNA expression of IGF-1R after $BRAF^{V600E}$ overexpression was not affected (Figure 17H). As the studies described above, we performed stability assays using CHX treatment of SKMEL19 melanoma cells with or without $BRAF^{V600E}$ overexpression or after AZD6244 treatment. The results indicate that after $BRAF^{V600E}$ overexpression, the stability of IGF-1R is increased (Figures 17I), whereas MEK inhibition using AZD6244 treatment of the cells did not significantly change protein stability of IGF-1R (Figures 17J). These data indicate that IGF-1R protein expression is positively regulated by BRAF and independent of pERK levels in melanoma cells, presumably by increased protein stability.

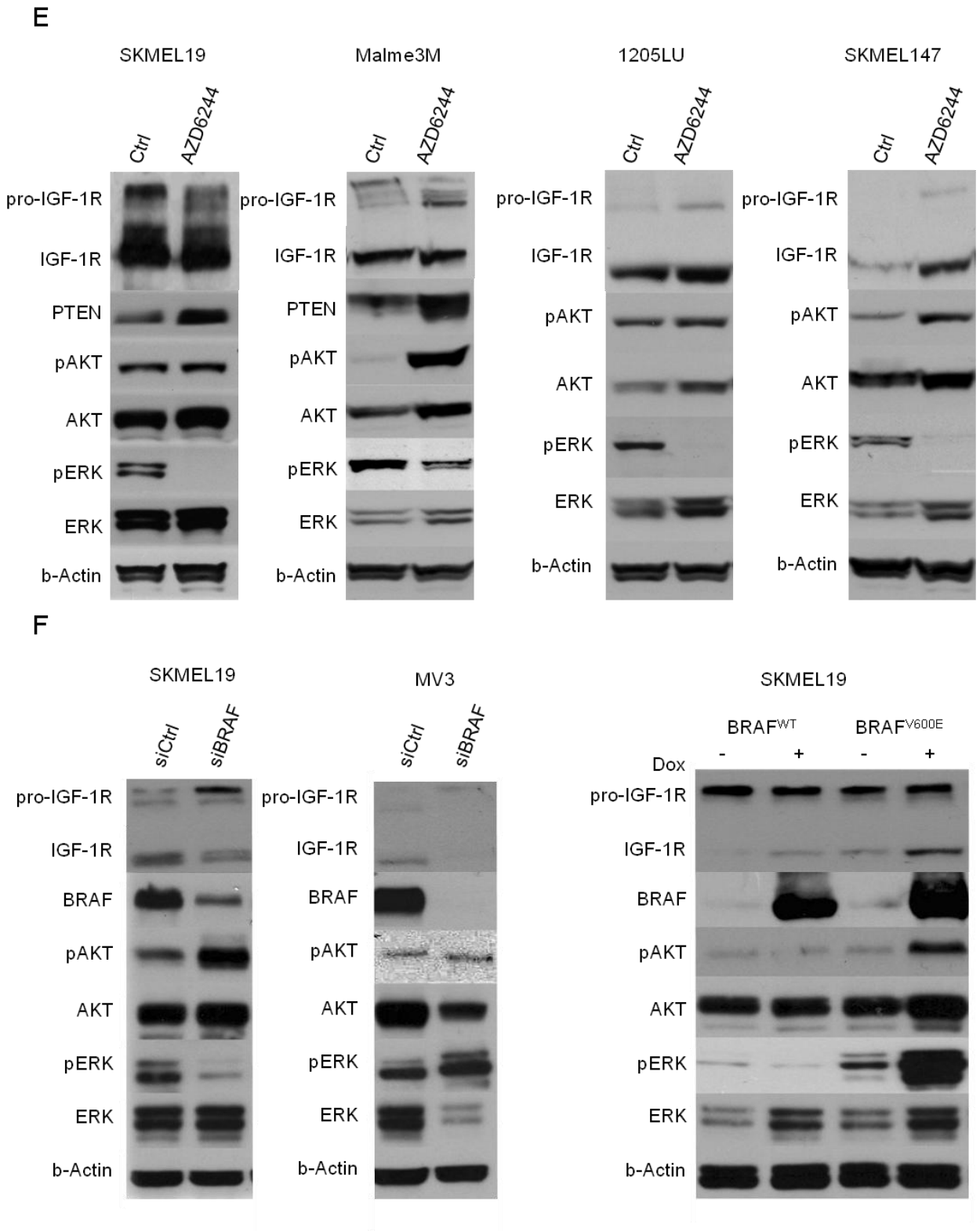

A
SKMEL19 (BRAF$^{V600E}$)
PLX4032 0 1 6 12 18 24(hrs)
pro-IGF-1R
IGF-1R
pAKT
AKT
pERK
ERK
β-Actin
MV3 (BRAF$^{WT}$)
PLX4032 0 1 6 12 18 24(hrs)
pro-IGF-1R
IGF-1R
pAKT
AKT
pERK
ERK
β-Actin
B
SKMEL19 (BRAF$^{V600E}$)
MV3 (BRAF$^{WT}$)
Isotype
Control
PLX4032
Count
Malme-3M (BRAF$^{V600E}$)
WM3211 (BRAF$^{WT}$)
IGF-1R(Log APC)

C


Rel. mRNA expression
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Malme3m
SKMEL19
A375
M14
MV3
WM3211
BRAFV600E
BRAFWT

D


SKMEL19
MV3
Isotype
Control
AZD
Count
500
375
250
125
0
100
75
51
26
1
IGF-1R(Log APC)


E
SKMEL19
Malme3M
1205LU
SKMEL147
Ctrl
AZD6244
pro-IGF-1R
IGF-1R
PTEN
pAKT
AKT
pERK
ERK
b-Actin
F
SKMEL19
MV3
SKMEL19
siCtrl
siBRAF
BRAF
BRAF^WT
BRAF^V600E
Dox
-
+

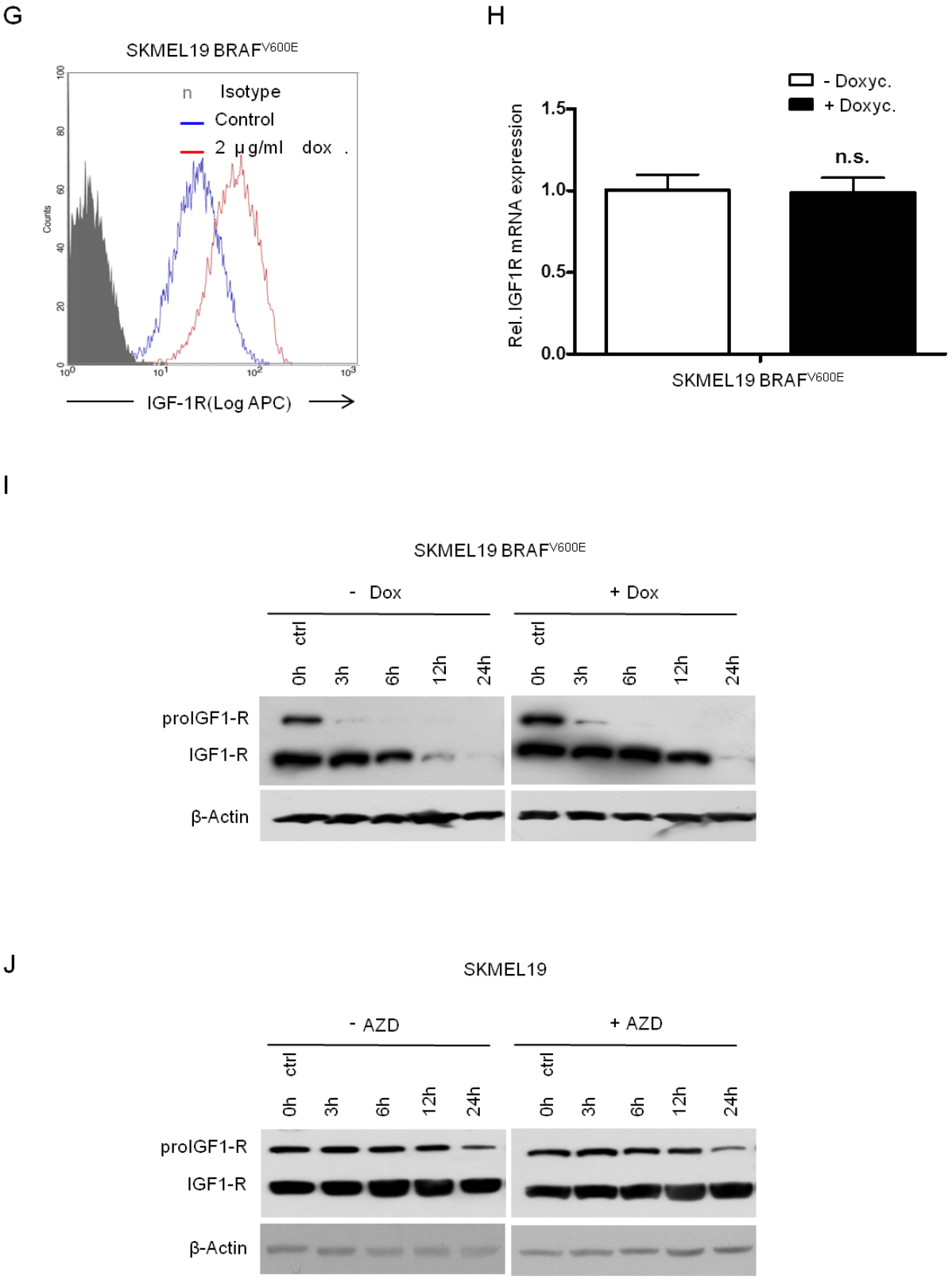



**Figure 17: BRAF enhances IGF-1R expression in a MAPK signalling activity independent manner.**

**(A**) The BRAF$^{V600E}$ melanoma cell line SKMEL19 and the BRAF wild-type melanoma cell line MV3 were treated with the BRAF$^{V600E}$ inhibitor PLX4032 (1 µM) for the indicated time points, and the cell lysates were harvested and immunoblotted with the indicated antibodies. **(B)** The surface expression of IGF-1R in indicated melanoma

cells in untreated (control) cells or cells treated for 24 hours with PLX4032 (1 µM) were assessed by flow-cytometric analyses. Filled grey field denotes the isotype control staining. **(C)** Shown is relative mRNA expression of the IGF-1R in the indicated melanoma cell lines expressing either wild-type or mutated $BRAF^{V600E}$ after treatment with PLX4032 (1 µM) for 6 hours. RNA expression was assessed by qRT-PCR and normalized to the respective untreated cells. (D) The surface expression of IGF1R in SKMEL19 or MV3 melanoma cells, which were either untreated or treated with the MEK inhibitor AZD6244 (4 µM) for 24 hours, by flow-cytometric analyses. Filled grey field denotes the isotype control staining. **(E)** The melanoma cell lines SKMEL19 and Malme3M (PTEN positive) and 1205LU and SKMel147 (PTEN negative) were treated with the MEK inhibitor AZD6244 (4 µM) for 24 hours. The cell lysates were harvested and immunoblotted with the indicated antibodies. **(F)** Left panels: scramble siRNA or siRNA against BRAF was transfected into SKMEL19 ($BRAF^{V600E}$) or MV3 (wild-type BRAF) melanoma cells. After 48 hours of transfection, the cell lysates were harvested and immunoblotted with the indicated antibodies. Right panel: either wild-type BRAF or $BRAF^{V600E}$ was overexpressed in SKMEL19 melanoma cells in an inducible manner by adding 4 µg/ml doxycycline. After 24 hours of transfection, cell lysates were harvested and immunoblotted with the indicated antibodies. **(G)** The surface expression of IGF-1R on SKMEL19 cells after 48 hours of inducible overexpression of $BRAF^{V600E}$ (red) compared to the non-induced cells (blue) were assessed by flow-cytometric analyses. Filled grey field denotes the isotype control staining. **(H)** Shown is relative mRNA expression of IGF1R after the induction of expression of $BRAF^{V600E}$ in SKMEL19 by adding doxycycline (2 µg/ml) for 48 hours. **(I)**The stability of IGF-1R was assessed in SKMEL19 melanoma cells after overexpression of $BRAF^{V600E}$. After 48 hours of the induction of $BRAF^{V600E}$ expression, cells were cultured in medium containing 150 µg/ml CHX to inhibit the protein de novo synthesis for the indicated time points. The cell lysates were harvested and immunoblotted with the indicated antibodies to detect IGF-1R and its pro-form in relation to beta-actin. **(J)** Stability assay of IGF1R described as in (I) but cells were pre-treated with the MEK inhibitor AZD6244 (4 µM) for 12 hours.

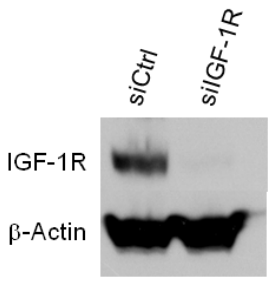
* The experiments of G-J have been done by Heike Niessner and Tobias Sinnberg.

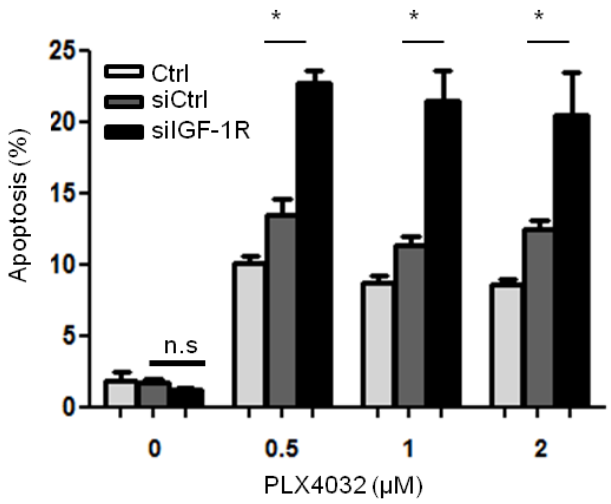
### 3.6 Activation in addition to up-regulation of the IGF-1R in melanoma cells increase therapy resistance

Despite of regulation of IGF-1R by BRAF, we addressed the functional relevance of the IGF-1R expression and activation concerning resistance towards the $BRAF^{V600E}$ inhibitor PLX4032. First, we down-regulated the IGF-1R in SKMEL19 melanoma cells using specific siRNA and analyzed the percentage of cells, which have undergone apoptosis after treatment with increasing doses of PLX4032. Intriguingly, down-regulation of the IGF-1R sensitized melanoma cells towards PLX4032 treatment (Figure 18A). Vice versa, over-expression of IGF-1R increased resistance towards PLX4032 by decreasing apoptosis. Interestingly, additional stimulation of the cells by IGF-1 furthermore increases resistance

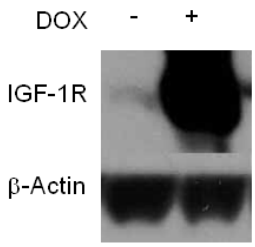
towards PLX4032 treatment (Figure 18B). This effect could be verified by a fluorescence based proliferation assay (Figure 18C). These data indicate that IGF-1R increases resistance towards PLX4032 and this effect is enhanced when the receptor is stimulated by its ligand IGF-1.

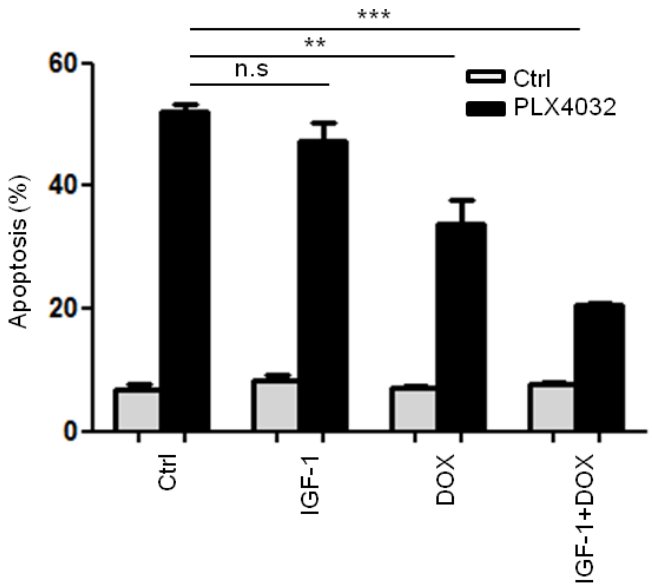
A




B



C

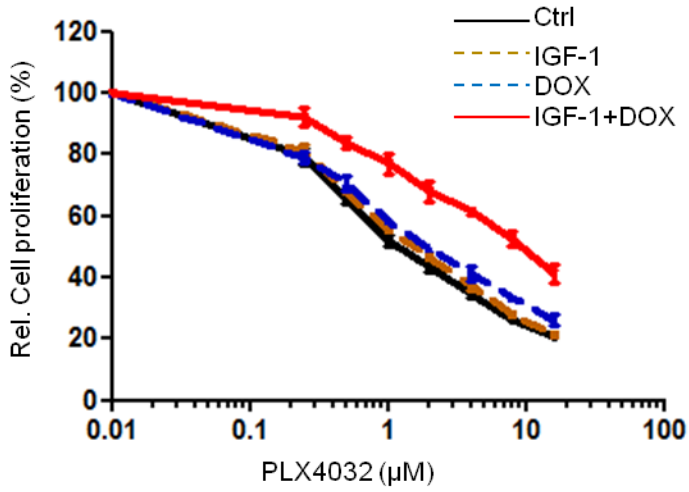


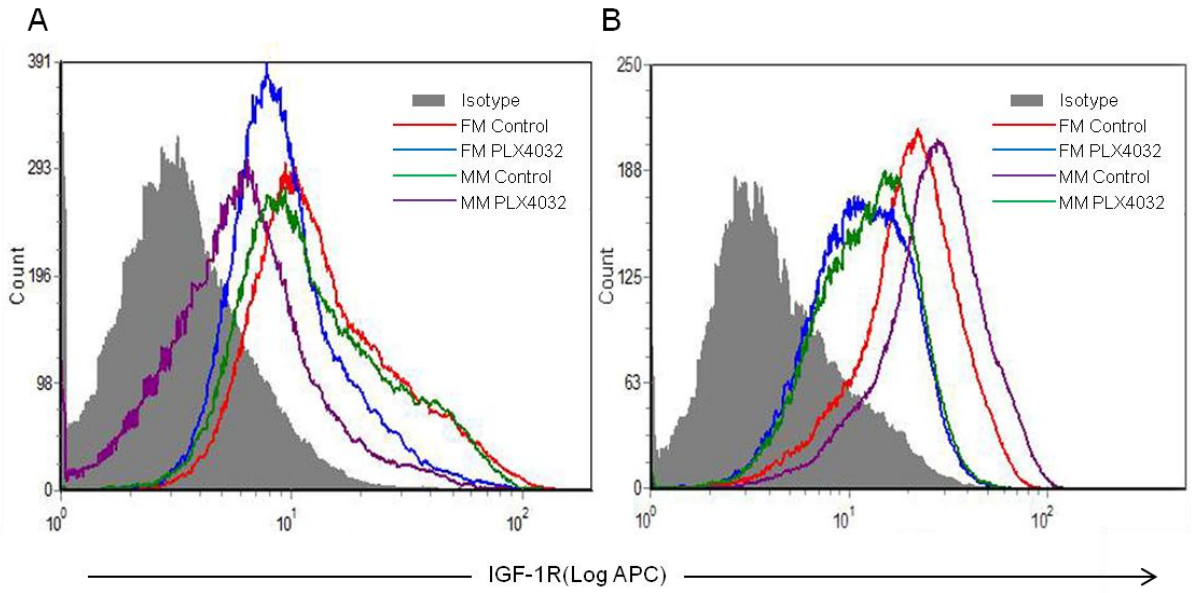

**Figure 18: Activation of up-regulated IGF-1R in melanoma cells increases therapy resistance.**

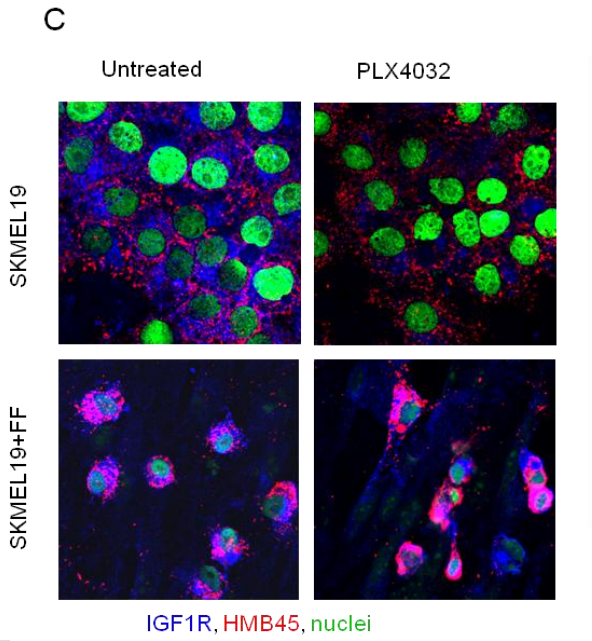
**(A)** Left: either scrambled siRNA or siRNA against IGF-1R was transfected into SKMEL19 melanoma cells. After 48 hours of transfection, cell lysates were harvested and immunoblotted with the indicated antibodies. Right: apoptosis was assessed by sub-G1 fraction of the cells. After 48 hours of transfection with either scrambled siRNA or siRNA against IGF-1R, the cells were then treated with increasing doses of PLX4032 for additional 2 days. The percentage of cells in sub-G1 cell cycle phase was determined with the help of PI staining and flow cytometric analyses. Data are presented as the mean of sub-G1 fractions from triplicate experiments. **(B)** Left: the overexpression of IGF-1R in SKMEL19 melanoma cells was induced by adding doxycycline (4 µg/ml) for 24 hours. The cell lysates were harvested and immunoblotted with the indicated antibodies. Right: apoptosis was assessed by sub-G1 fraction of the cells. The overexpression of IGF-1R in SKMEL19 melanoma cells was induced by adding doxycycline (4 µg/ml) for 24 hours. The cells were then treated with PLX4032 (1µM) alone or in combination with 100 ng/ml IGF-1 for additional 72 hours. The percentage of cells in sub-G1 cell cycle phase was determined with the help of PI staining and flow cytometric analyses. Data are presented as the mean of sub-G1 fractions from triplicate experiments. Compared to control, P value of IGF-1 is 0.0706. P value of doxycycline is 0.0015. P value of IGF-1 with doxycycline is <0.0001. **(C)** The cell viability was assessed by MUH assay after treatment with increasing doses of PLX4032 alone or in combination with 100 ng/ml IGF-1 for 72 hours. Before PLX4032 treatment IGF-1R expression was either induced to overexpress in cells by addition of 4 µg/ml doxycycline for 24 hours or not.

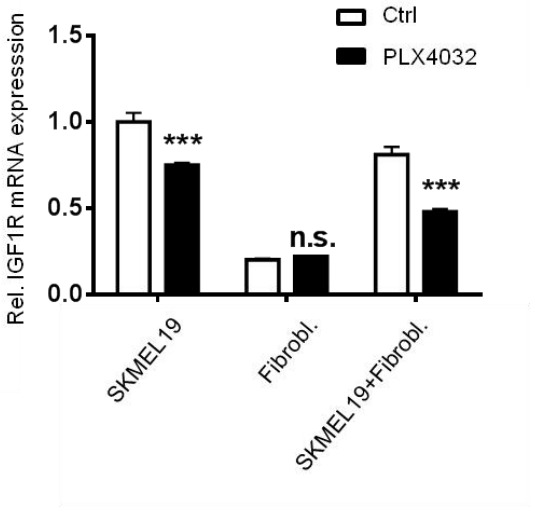
## 3.7 Dermal fibroblasts mediate up-regulation of the IGF-1R by cell-cell contact and increase therapy resistance

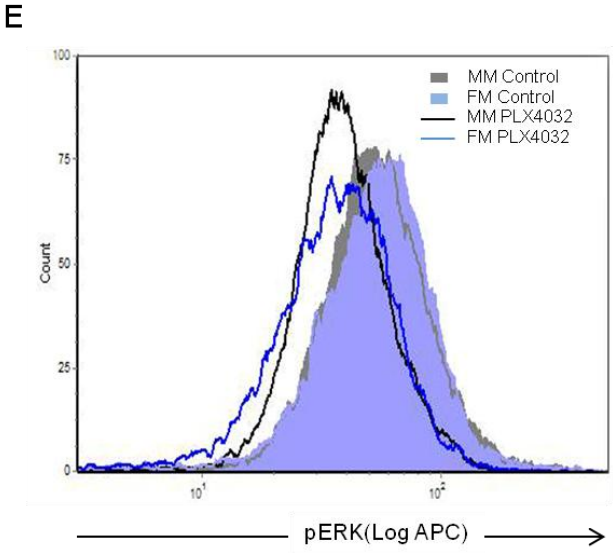
Although stimulation with IGF-1 alone is not enough to protect melanoma cells from PLX4032 treatment, we reasoned that a way how to achieve innate therapy resistance of melanoma cells in a physiological situation must be mediated by activation and up-regulation of the

IGF-1R by micro-environmental signals. Melanoma cells can stimulate the recruitment of fibroblasts and activate them, resulting in melanoma cell growth by providing both structural (extracellular matrix proteins), and chemical support (growth factors), and conferring treatment resistance to melanoma cells as well (Flach, Rebecca et al. 2011). Therefore, we proposed that interaction of melanoma cells with human primary fibroblasts modulates IGF-1R expression and resistance towards PLX4032. To confirm this hypothesis, we co-cultured GFP tagged SKMEL19 cells with dermal fibroblasts. After treatment with PLX4032, expression of the IGF-1R on the cell surface of melanoma cells was analyzed by FACS. We found that co-cultivation of SKMEL19 melanoma cells with human primary dermal fibroblasts slightly increases surface expression of the IGF-1R (Figure 19A and 19C), but did not activate MAPK signaling (Figure 19E). More importantly, whereas PLX4032 treatment of melanoma cells reduced surface IGF-1R expression as shown before, co-cultivation of melanoma cells with primary fibroblasts resulted in a significantly reduced down-regulation of the IGF-1R after PLX4032 treatment (Figure 19A and 19C). This effect is cell-cell contact dependent and not mediated by fibroblast-derived soluble factors because we do not see the effect when we inhibit cell-cell contact by co-culture in a Boyden chamber (Figure 19B). Moreover, the effect was less pronounced on RNA level suggesting again a posttranscriptional mechanism (Figure 19D). These data indicate that cell-cell contact of human melanoma cells with dermal fibroblasts stabilizes IGF-1R surface expression. Interestingly, co-cultivation of melanoma cells with human primary fibroblasts not only in monolayer (Figure 19F) but also in 3-dimensional spheroids (Figure 19H) enhanced significantly the resistance towards PLX4032 treatment in melanoma cells. The increased resistance is partly mediated by the IGF-1R as seen after down-regulation of IGF-1R by siRNA in the monolayer culture system (Figure 19G). These data indicate that primary human fibroblasts are able to stabilize IGF-1R expression on melanoma cells by cell-cell contact and confer enhanced resistance towards PLX4032.


A
B
Isotype
FM Control
FM PLX4032
MM Control
MM PLX4032
Count
IGF-1R(Log APC)


C
Untreated
PLX4032
SKMEL19
SKMEL19+FF
IGF1R, HMB45, nuclei


D
Ctrl
PLX4032
Rel. IGF1R mRNA expresssion
***
n.s.
***
SKMEL19
Fibrobl.
SKMEL19+Fibrobl.


E
MM Control
FM Control
MM PLX4032
FM PLX4032
Count
pERK(Log APC)

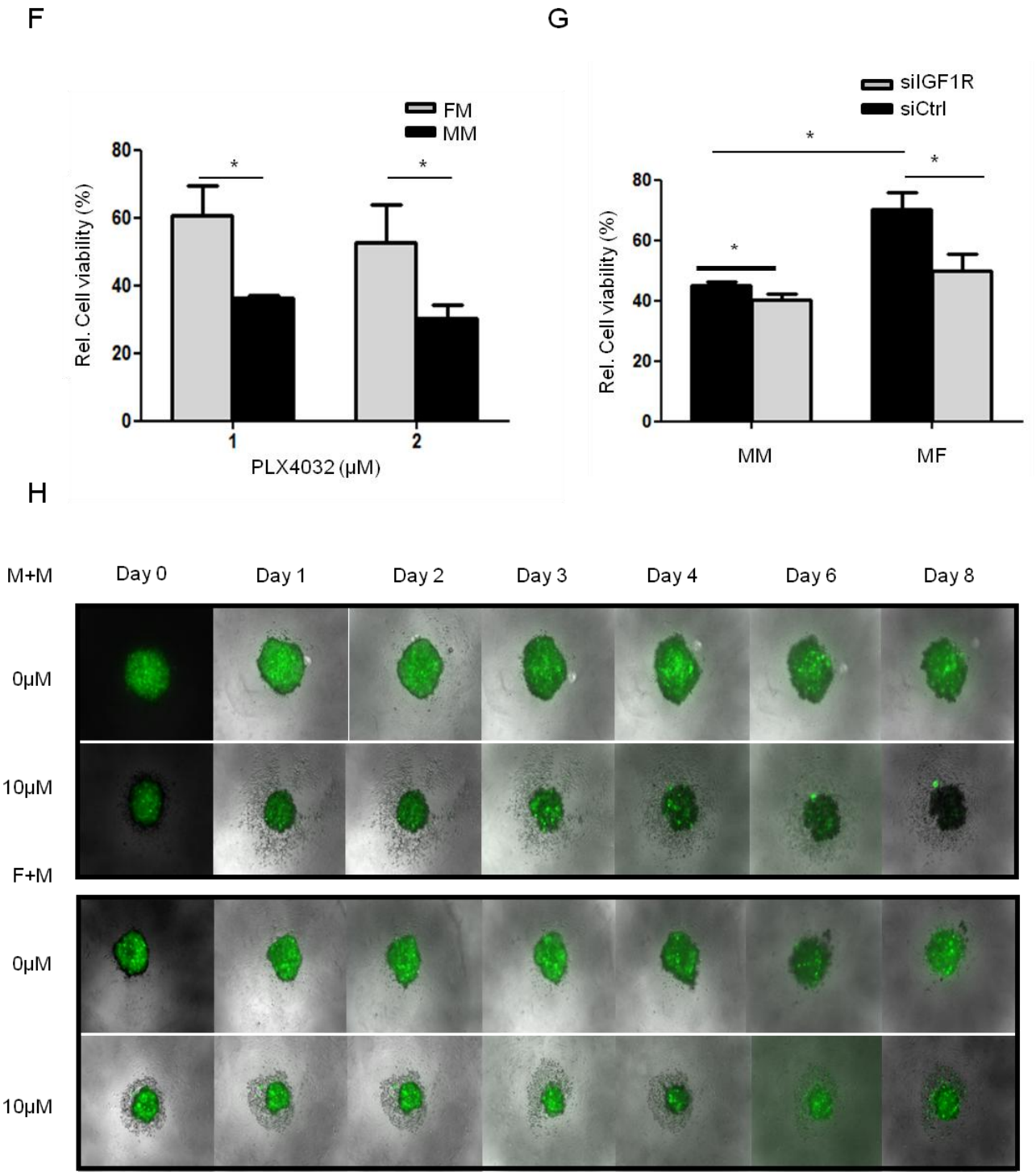



**Figure 19: Dermal fibroblasts mediate up-regulation of the IGF-1R by cell-cell contact and increase therapy resistance.**

**(A, B)** The surface expression of IGF1R in GFP-expressing SKMEL19 melanoma cells by flow-cytometric analyses. In (A), the cells were pre-cultivated for 24 hours with primary human dermal fibroblasts (FM) or not (MM), and then were either untreated or treated with PLX4032 (1 µM) for additional 24 hours. In (**B**), Co-cultivation was done in Boyden chambers to inhibit fibroblast-melanoma cell contact, but which allowed secreted factors to reach their targets. After 24 hours of co-cultivation, the cells were either untreated or treated for 24 hours with PLX4032 (1 µM). Filled grey field denotes isotype control staining. (**C**) $3x10^4$ SKMEL19

melanoma cells were cultured alone or with fibroblast on fibronectin coated (50 µg/ml) chamber slides overnight, and then were treated with PLX4032 (2 µM) for 24 hours. After that, the Cells were fixed and stained using anti-IGF-1R (blue), HMB45 (red) for the detection of melanocytic cells, and analysed using a confocal laser-scanning microscope. Nuclei were stained with YOPRO (green). (**D**) Shown is relative mRNA expression of IGF1R. Cells were treated as in (A), harvested and prepared for qRT-PCR. For statistics Two-way ANOVA analysis has been done. (**E**) The cytoplasmic pERK in GFP-expressing SKMEL19 melanoma cells by flow-cytometric analyses. The cells were pre-cultivated for 24 hours with primary human dermal fibroblasts (FM) or not (MM), and then were either untreated or treated with PLX4032 (1 µM) for additional 24 hours. **(F, G)** The cell viability of GFP-expressing SKMEL19 melanoma cells was assessed by measuring fluorescence intensity using a fluorometer. In (F), the melanoma cells were either cultured alone (MM) or with primary human dermal fibroblasts for 24 hours, and then treated with PLX4032 (1µM or 2µM) for additional 72 hours. Data are presented as the mean of relative viability compared to untreated cells set as 1 from triplicate experiments. In (G), scramble siRNA or siRNA against IGF-1R was transfected into GFP-expressing SKMEL19. After 24 hours of transfection, the cells were cultured alone (MM) or with primary human dermal fibroblasts (FM) for 24 hours, and then the cells were treated with PLX4032 (1 µM) for additional 72 hours. Data are presented as the mean of relative viability compared to untreated cells set as 1 from triplicate experiments. **(H)** Shown is a three-dimensional spheroid assay. GFP-expressing SKMEL19 melanoma cells alone (M+M) or with primary human dermal fibroblasts (F+M) were seeded to form spheroids in 1% Agarose coated 96 well plates, and then the spheroids were treated with PLX4032 (10 µM) up to 8 days. PLX4032 was refreshed every 3 days. Cell viability was measured by determination of GFP-expressing SKMEL19 melanoma cells using fluorescence microscopy.

# 4 Discussion:

The IGF-1 signaling pathway plays a relevant role in regulating cellular proliferation and apoptosis. There is now considerable evidence that the risk of several common cancers increases when the level of circulating IGF-1 is high (Pollak, Schernhammer et al. 2004). The targeting of IGF-1R by various therapeutic strategies has demonstrated encouraging anti-neoplastic activity in laboratory models, which justifies the expansion of clinical trials of several novel drug candidates (Pollak, Schernhammer et al. 2004; Riedemann and Macaulay 2006; Tao, Pinzi et al. 2007). In melanoma, the IGF-1R is overexpressed compared with benign naevi (Kanter-Lewensohn, Girnita et al. 2000). IGF-1R antisense oligonucleotides have been shown to inhibit melanoma cell survival *in vitro* and growth of FO-1 melanoma xenografts in nude mice (Resnicoff, Coppola et al. 1994). Knockdown of IGF-1R is capable of inducing significant survival inhibition and enhancement of apoptosis, regardless of BRAF mutation status (Maloney, McLaughlin et al. 2003; Yeh, Bohula et al. 2006; Macaulay, Middleton et al. 2013). The importance of IGF-1R expression and activity was stressed again in therapy-resistance of malignant melanoma against the $BRAF^{V600E}$ inhibitor PLX4032 (Villanueva, Vultur et al. 2010). These findings suggest that the IGF-1R is an attractive target for melanoma treatment.

IGF-1R is widely expressed on neoplastic and normal tissues. Although receptor levels in cancers are sometimes higher than the levels seen in normal tissues, gene amplification associated with large increases in receptor number is rare (Tao, Pinzi et al. 2007; Pollak 2012). Therefore, understanding the molecular mechanisms regulating IGF-1R expression and activity in melanoma cells is indispensable.

In this study, we show that the expression of IGF-1R is upregulated in melanoma cells. PTEN regulates IGF-1R expression mainly on posttranscriptional level in a phosphatase dependent manner, and PTEN loss melanoma cells escape IGF-1R blockade by decreased IGF-1R expression after treatment with IGF-1R inhibitor. Moreover hyperactivation of BRAF elevates IGF-1R expression by increasing its stability. PLX4032 treatment decreases IGF-1R expression, which play a relevant role for the inhibitory function of PLX4032. In addition, we

also point out that heterocellular contact of melanoma cells with dermal fibroblasts stabilizes IGF-1R expression on melanoma cell membrane, which confers melanoma initial resistance to PLX4032.

### 4.1 IGF-1R expression correlates with expression of PTEN in human melanoma cells

PTEN is either lost or impaired in up to 30% of melanomas (Teng, Hu et al. 1997; Tsao, Goel et al. 2004). Compared to primary melanomas, loss of PTEN expression is mainly found in melanoma metastases. It cooperates with the $BRAF^{V600E}$ mutation seen in about half of all melanoma samples to promote melanoma metastases formation (Dankort, Curley et al. 2009). Moreover, as a negative regulator of PI3K/AKT pathway, loss of PTEN leads to increased activity of PI3K/AKT pathway (Keniry and Parsons 2008; Aguissa-Toure and Li 2012). In line with it, we show that loss of PTEN results in higher activity of PI3K/AKT pathway than that in PTEN expressing melanoma cells.

Our results also show a positive correlation of PTEN and IGF-1R expression in several human melanoma cell lines already on protein and RNA level. Modulation of PTEN expression in PTEN loss and PTEN expressing melanoma cells confirms its direct regulatory effect on IGF-1R expression. Further we describe that the regulation of IGF-1R by PTEN is not dependent on its negative regulatory function of PI3K activity, which is differ from the report in glioblastoma cell lines by Lackey et al.(Lackey, Barnett et al. 2007). It could be due to tumor cell-specific mechanisms to modulate IGF-1R expression. Moreover, we demonstrated that neither of single nor both phosphatase-deficient mutants are able to increase expression of the IGF-1R. Although overexpression of wild-type PTEN is able to induce an up to two-fold increase in IGF-1R mRNA expression, this cannot explain the more than 7-fold higher protein expression of the IGF-1R. Therefore, our results point out that PTEN is able to increase IGF-1R expression in melanoma cells mainly on a posttranscriptional level by its lipid and protein phosphatase activity. Further our study shows that the stability of the IGF-1R and its pro forms before and after PTEN induction is not different. Hence, the PTEN phosphatase-dependent increase in IGF-1R expression in melanoma cells is not the result of increased stabilization of the IGF-1R protein. By

endocytosis IGF-1R is incorporated into clathrin- or caveolin-coated vesicles, by which the IGF-1R is recycled back to the cell surface or degraded by the proteasome and lysosomal pathways (Girnita, Girnita et al. 2003; Sehat, Andersson et al. 2007; Sehat, Andersson et al. 2008). This process is regulated by phospho-tyrosine motifs of IGF-1R (Girnita, Worrall et al. 2014). As a phosphatase, PTEN possess both lipid phosphatase and protein phosphatase activity (Shi, Paluch et al. 2012). It indicates that PTEN has potential to influence IGF-1R recycling by regulating phosphorylation of tyrosine, the signal of internalization, to modulated IGF-1R expression. Our results also confirmed that the expression of wild-type PTEN, but not of the mutant forms, increased surface expression of the IGF-1R. Therefore our results demonstrated that PTEN regulated IGF-1R expression might be achieved by a combination of enhanced IGF-1R transcription and increased protein shuttling.

Interestingly, we also found that over-expression of PTEN in PTEN negative melanoma cell lines in a short time does not affect survival and proliferation of melanoma cells contrary to what is seen in other tumor systems (Aguissa-Toure and Li 2012). On one side it might be that we over-express PTEN using an inducible expression system, which regulates the amount of PTEN in such a moderate level that it is less toxic effect to melanoma cells; on the other side it might also be that melanoma cells have a better counter-regulatory mechanism than other tumor cells due to the constitutive activation of MAPK signaling.

Interestingly, we show that PTEN expression determines sensitivity toward IGF-1R inhibition. Melanoma cells, which had lost PTEN expression and low IGF-1R expression, are more resistant to IGF-1R inhibition. In the presence of PTEN, it counteracts the IGF-1R inhibitor induced decreased expression of IGF-1R and increases sensitivity toward IGF-1R inhibition. IGF-1R inhibitors are used in several clinical trials. Therefore, this finding has important therapeutic implications, suggesting that the dose of IGF-1R inhibitor used should be adjusted depending on the PTEN expression levels in the tumor.

## 4.2 The regulation of IGF-1R expression is independent on PI3K and AKT activity in melanoma cells

Previous studies found that IGF-1R activity and expression is negative modulated by PI3K/AKT pathway activity, which is in part due to mTORC1 inhibition and in part to a FOXO-dependent activation of receptor expression (Chandarlapaty, Sawai et al. 2011). Our result also shows that IGF-1R expression negatively correlates with activation of PI3K/AKT signaling in human melanoma cells. However, we found that transient inhibition of PI3K by different inhibitors has no effect on expression of IGF-1R. We also modulated AKT activity by modulating AKT3 expression, the dominant isoform in melanoma (Stahl, Sharma et al. 2004). As same as inhibition of PI3K, it has also slightly effect on IGF-1R expression. The reason why the feedback mechanism doesn´t work in melanoma cells is still elusive. One explanation might be that there seems to be tumor cell-specific mechanisms to modulate IGF-1R expression.

## 4.3 BRAF enhances IGF-1R expression independent on MAPK signaling activity

A number of novel BRAF or MEK inhibitors have been developed and the most promising inhibitors used at the moment in the clinic are PLX4032, which inhibits specifically $BRAF^{V600E}$, and the selective MEK1/2 inhibitors selumetinib (AZD6244, ARRY-142886) and trametinib. Phase 1-3 clinical trials of the BRAF kinase inhibitor PLX4032 have shown impressive response rates of more than 50% in patients with metastatic melanoma with the $BRAF^{V600E}$ mutation (Flaherty, Puzanov et al. 2010; Chapman, Hauschild et al. 2011). However, PLX4032-treated melanoma patients rapidly developed therapy resistance within 7 months. Therefore understanding the resistance mechanism is necessary. Recently, over-expression of the IGF-1R was shown to be a key mechanism conferring secondary resistance towards the $BRAF^{V600E}$ inhibitor PLX4032 (Villanueva, Vultur et al. 2010). However another group showed that stimulation with IGF-1, the cognate ligand, was not able to protect melanoma cells from PLX4032 treatment (Straussman, Morikawa et al. 2012). The paradox results suggest that there must be different intrinsic intracellular regulatory mechanisms, by which the expression and activity of IGF-1R signalling are regulated in PLX4032 sensitive and

resistant melanoma cells. Our results show that inhibition of $BRAF^{V600E}$ by PLX4032 reduced IGF-1R expression on transcription and protein level in a MAPK pathway independent manner. Moreover localization of IGF-1R on cell membrane surface, where IGF-1R takes its function to transduce signals downstream, is also dramatically decreased after PLX4032 treatment. Furthermore, the evidences that down-regulation of IGF-1R sensitizes melanoma cells to PLX4032 treatment, and over-expression of IGF-1R in melanoma cells using inducible virus system increases resistance towards PLX4032 treatment, which is enhanced by activation of IGF-1R with its ligand IGF-1, stresses that down-regulation of IGF-1R plays a key role on the function of PLX4032. In addition, we also confirm that $BRAF^{V600E}$ itself can increase stability of IGF-1R and enhance its localization on cell membrane in a MAPK activity independent manner. Our results probably confer an explanation to the paradox function of IGF-1R in PLX4032 sensitive and resistant melanoma cells. But how the chronic treatment of PLX4032 induces IGF-1R over-expression has still to be elucidated.

### 4.4 Dermal fibroblasts mediate up-regulation of the IGF-1R by cell-cell contact and increase therapy resistance

Although a few studies show that melanoma cells isolated from PLX4032 resistant patient have additional mutation (Johannessen, Boehm et al. 2010; Nazarian, Shi et al. 2010; Shi, Hong et al. 2014; Van Allen, Wagle et al. 2014), the main reason for acquired resistance of melanoma to PLX4032 treatment is reactivation of MAPK by formation of hetero-dimer of BRAF with ARAF or CRAF, or by elevated expression of RTK (Nazarian, Shi et al. 2010; Poulikakos and Rosen 2011). It indicates that the origin of resistant melanoma cells are not due to drug selection of heterogeneous subpopulation of tumor cells, but due to reprogramming of some of melanoma cells surviving from PLX4032 treatment, when exposed to PLX4032. Therefore, understanding of the mechanism by which melanoma cells acquire initial resistance to PLX4032, give a chance to overcome the resistance of melanoma cells to PLX4032 treatment. Recently, one study demonstrated that the transcriptional profiles differed in RAF inhibitor-sensitive and inhibitor-resistant $BRAF^{V600}$-mutant melanomas (Konieczkowski, Johannessen et al. 2014). Most drug-sensitive cell lines and

patient biopsies showed high expression and activity of the melanocytic lineage transcription factor MITF, while intrinsically resistant cell lines and biopsies displayed low MITF expression but higher levels of NF-κB signaling and the receptor tyrosine kinase AXL. In addition to intrinsic factors, the tumor micro-environment confers innate resistance to therapy (Gkretsi, Stylianou et al. 2015). One study showed that the secretion of hepatocyte growth factor (HGF) by tumor stromal cells resulted in activation of the HGF receptor (MET), and sequently activated the MAPK and PI3K/AKT pathways, thus conferring melanoma cells resistance to RAF inhibition (Straussman, Morikawa et al. 2012). It was also showed that melanoma cells can recruit and activate fibroblasts, which provide both structural (extracellular matrix proteins) and chemical support (growth factors), therefore resulting in melanoma cell growth and drug resistance (Flach, Rebecca et al. 2011). In line with it, Hirata et al. showed that melanoma-associated fibroblasts stimulate matrix production/remodeling, and, consequently, survival signaling in melanoma cells via β1-integrin, Src, and FAK, which can drive resistance to the BRAF inhibitor PLX4720 (Hirata, Girotti et al. 2015). Primary fibroblasts mediated initial resistance to PLX4032 treatment is also confirmed in our study. Although it showed that fibroblast-derived IGF-1 could not mediate resistance towards BRAF inhibition (Nazarian, Shi et al. 2010; Villanueva, Vultur et al. 2010; Straussman, Morikawa et al. 2012), interestingly we show that primary fibroblasts are able to stabilize IGF-1R expression on the cell surface by yet unknown cell-cell interaction. Destruction of heterocellular interaction or down-regulation of IGF-1R expression by small interfering siRNA in melanoma cells is able to diminish fibroblast mediated PLX4032 resistance. There has been increasing recognition, that integrin activation is able to modify the phosphorylation and signaling from the IGF-1R and subsequently the biological outcomes (Maile and Clemmons 2002; Ivaska and Heino 2010). The O'Connor group described that the RACK1 scaffolding protein, known to interact with β1 integrin, binds to IGF-1R, and affects Shc/Grb2 downstream signaling (Hermanto, Zong et al. 2002). Further, the O'Conner group demonstrated that the mutually exclusive association of RACK1 with phosphatase PP2A or β1 integrin is controlled by IGF-1 stimulation (Kiely, O'Gorman et al. 2006). Recently, the Takada group further stressed cross-talk between the

two pathways, in accord with the co-localization of IGF-1R and integrins together at focal adhesion complexes. They found that IGF-1 ligand binds directly to αvβ3 integrin and α6β4 integrin, thus influencing IGF-1R signaling (Saegusa, Yamaji et al. 2009), including sustaining of cells during anchorage independence (Fujita, Ieguchi et al. 2012). The results indicate that the interaction of IGF-1R with integrins might be a key regulator for fibroblast mediated therapy resistance. But it has to be shown whether melanoma-fibroblast adhesion is able to increase IGF-1R stability; whether PTEN is involved in this process; and how IGF-1R and its activity influence the signaling mediated by FAK.

Taken together, according our results we propose the network of IGF1R regulation in melanoma as shown in figure 20. Both tumor cell intrinsic mechanisms with IGF-1R stabilization by hyperactivation of BRAF and/or PTEN and microenvironmental influences with IGF-1R stabilization by melanoma cell-fibroblast contact enhance expression and activation of IGF-1R promoting melanoma progression and therapy resistance.

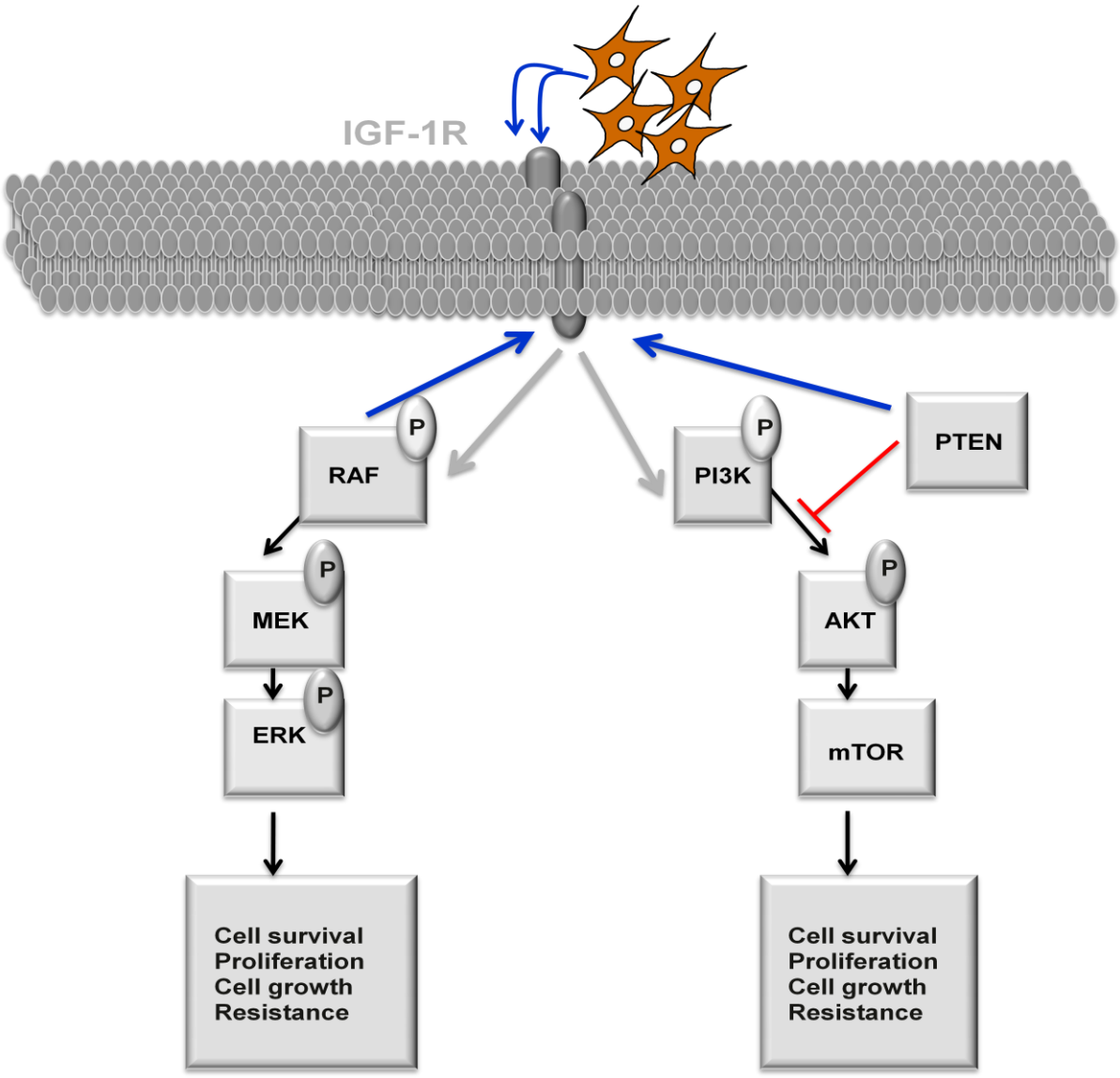



**Figure 20: Proposed model of the mechanisms regulating IGF-1R expression in melanoma cells.**
IGF-1R is regulated in melanoma cells by a combination of tumor cell intrinsic mechanisms as stabilization of the IGF-1R either by hyper-activation of BRAF or probably protein trafficking by PTEN as well as micro-environmental heterocellular contacts with fibroblast. Enhanced expression and activation of IGF-1R lead to melanoma progression and therapy resistance.

# 5 Outlook

We showed that PTEN influences IGF-1R expression and by this therapy resistance in melanoma. It has still to be solved what are the molecular mechanisms behind this. Further we will also investigate if PTEN can influence expression of other RTKs, such as EGFR and Met, which play a relevant role in melanoma progression and therapy resistance. Moreover, it has still to be solved what are the molecular mechanisms involved in fibroblast-melanoma cellular contact induced enhancement of IGF-1R expression. Interestingly, it has been shown that the IGF-1R can translocate to the nucleus and auto-regulates its own expression in breast cancer cells (Sarfstein, Pasmanik-Chor et al. 2012). Also, nuclear IGF-1R has been considered to be involved in a switch in oncogene-induced transformation at advanced stages of the disease. In addition, nuclear IGF-1R is complexed with LEF-1/TCF at the human cyclin D1 promoter to increase expression of cyclin D1, hence contributing to neoplastic transformation. It seems that nuclear IGF-1R may serve as a potential prognostic biomarker for successful therapy, therefore it has to be seen whether the IGF-1R is also present in the nucleus in melanoma cells and what the clinic relevance it takes.

# 6 Summary

Among RTKs, the IGF-1R plays a relevant role in cancer development. IGF-1R and its natural ligands regulate multiple cellular functions, including cellular proliferation, survival, anchorage independent growth, tumor neovascularization, migration, invasion, and metastasis. But in clinical settings, the mechanism of aberrant IGF-1R is still not clear: the receptor does not show intrinsic receptor abnormalities, and the large increases in receptor number are rarely associated with gene amplification (Tao, Pinzi et al. 2007; Pollak 2012). Therefore, understanding the molecular mechanisms regulating IGF-1R expression and activity in melanoma cells is indispensable. Our study showed that in melanoma cells IGF-1R expression is regulated by tumor cell intrinsic mechanisms and that hyperactivation of BRAF and the phosphatase activity of PTEN increase IGF-1R expression. Besides this, heterocellular contact of melanoma cells with dermal fibroblast stabilizes IGF-1R expression on the cell membrane. Enhanced expression and activation of IGF-1R are able to promote melanoma therapy resistance against PLX4032 treatment. Interestingly, PTEN-negative melanoma cells escape IGF-1R blockade by a downregulation of IGF-1R expression, implicating that only in melanoma patients with PTEN-positive tumors treatment with IGF-1R inhibitors would be a suitable strategy to combat therapy resistance. Our study emphasizes that microenvironmental and tumor cell-intrinsic mechanisms regulate IGF-1R expression and thus modulate sensitivity to targeted therapies.

## Zusammenfassung:

Der IGF-1R spielt unter den RTKs mit die wichtigste Rolle in der Krebs-Entwicklung. Der IGF-1R und seine Liganden regulieren verschiedenste Zellfunktionen, die wesentlich für die Entstehung des malignen Phänotyps sind; einschließlich Zellproliferation, Überleben, Wachstum unabhängig von Zellkontakthemmung, Tumorneovaskularisierung sowie Migration, Invasion und Metastasierung der Tumorzellen. In der Klinik konnte jedoch bisher kein klarer Mechanismus von dysreguliertem IGF-1R gefunden werden: Der Rezeptor weist keine intrinsische fehlerhafte Veränderung auf und auch eine erhöhte Expression des Rezeptors durch Genamplifikation ist äußerst selten. Deshalb ist das Verständnis der molekularen Mechanismen, die die Expression und Aktivität des IGF-1Rs in Melanom-Zellen regulieren, unentbehrlich.

Unsere Studie zeigt, dass die Expression von IGF-1R in Melanomzellen durch einen intrinsischen Mechanismus reguliert wird; außerdem spielen die erhöhte Expression des IGF-1R durch Hyperaktivierung von BRAF sowie der Phosphatase-Aktivität von PTEN eine Rolle. Darüber hinaus stabilisiert der Kontakt von Melanomzellen mit dermalen Fibroblasten die Expression von IGF-1R auf deren Zellmembran. Erhöhte Expression und Aktivität von IGF-1R können Resistenz gegenüber der Behandlung mit z.B. BRAF-Inhibitoren wie PLX4032 vermitteln. Interessanterweise können PTEN-negative Melanomzellen der negativen Wirkung der IGF-1R Inhibition durch eine verminderte Expression des IGF-1R entgehen. Das bedeutet, dass nur in Melanompatienten mit einem PTEN-positiven Tumor die Behandlung mit Hemmstoffen gegen IGF-1R eine passende Strategie darstellt, um die Therapieresistenz zu bekämpfen. Unsere Studie hebt hervor, dass das Mikromilieu des Tumors und Tumorzell-intrinsische Mechanismen die Expression des IGF-1R regulieren und dadurch das Therapieansprechen auf zielgerichtete Hemmstoffe beeinflussen können.

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