The effect of the PKM2-EGFR axis on glioblastoma multiforme growth

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1 Introduction

1.1 Glioblastoma multiforme

Glioblastoma multiforme (GBM) is the most common malignant brain cancer in adults. In the vast majority of cases it arises from a cerebral hemisphere. The term glioblastoma results from the concept, that glioblasts are the cells from which the tumour originates. The term multiforme is used to describe the appearance of the tumour, which is characterized by frequent occurrence of hypoxic zones, multifocal necrosis, neovascularisation, haemorrhage, and cysts (Bailey & Cushing, 1926, Pistollato et al., 2010). Additionally, both the cells and the nuclei of GBM cells show highly polymorphic appearance. GBM is classified as a WHO grade 4 tumour, as it exhibits the traits of a highly aggressive, hyperproliferative and invasive tumour (Louis et al., 2021).

Although the peak incidence lies between 55 and 65 years, GBM may occur at any age (Smith, Ironside, 2007). There is no identified method for preventing the occurrence of GBMs (Gallego, 2015), and although GBMs are not prone to forming metastasis, the prognosis for the patients is still very poor with 5-year survival rates of less than 5%. Up to date, this outcome has not been improved by targeted therapy. An extensive treatment regimen involving surgery, radiotherapy and temozolomide chemotherapy prolongs life to approximately 14 months on average. Without any therapy, the patients usually have just a few months left to live (Johnson, O'Neill, 2012, Le Rhun et al., 2019).

1.1.1 Molecular characterisation of GBM subgroups

According to Verhaak and colleagues, GBM can be classified into four subtypes which feature unique genetic and transcriptome signatures:

1. Within the classical subtype nearly all (>95%) tumours contain amplified or mutated genes of the epidermal growth factor receptor (EGFR). This is often due to the amplification of chromosome 7.

2. The neural subtype expresses markers typical of normal neuron cells. Besides that, tumours in this category contain amplified receptor tyrosine kinases (RTKs), including amplified EGFR.

3. The mesenchymal subtype typically contains alterations of neurofibromin 1 (NF1) and a high expression of the mesenchymal markers chitinase 3-like protein 1 (CHI3L1) and the RTK MET.

4. The proneural subtype shows features typical for low-grade glioma. It is often characterized by alterations of p53, the a-type platelet-derived growth factor receptor (PDGFRA), MET, and the isocitrate dehydrogenase (IDH) (Verhaak et al., 2010). In the latest WHO 2021 classification these tumours are not referred to as glioblastomas anymore, but form the distinct group of IDH-mutated astrocytomas (Louis et al., 2021).

The diagnostic/prognostic markers for GBM include the overexpression and amplification of EGFR, the mutation of isocitrate dehydrogenase enzyme 1/2 (IDH1/2) or the telomerase reverse transcriptase (TERT1) promoter, genetic losses of chromosomes, and the epigenetic methylation status of the gene promoter for O6-methylguanine-DNA methyltransferase (MGMT) (Aquilanti et al., 2018). In the current WHO classification, however, GBM is characterised by IDH1/2 wild-type status (Louis et al., 2021). Interestingly, the genetic alterations in GBMs pool into three main signalling pathways. The Retinoblastoma (Rb) signalling is modified in around 78% of the tumours, and both the p53 pathway and the RTK/RAS/PI3K signalling are enhanced in approximately 85% of the GBMs (Aldape et al., 2015).

1.1.2 The metabolism of GBM

Dysfunctional tumour suppressor proteins such as p53 and Rb, the RTK/RAS/PI3K pathway and hypoxia all independently lead to typical changes in glucose metabolism. Among these, the RTK-mediated activation of the transcription factor c-Myc is the most potent driver of glycolytic metabolism (Masui et al., 2019). Those changes in glucose consumption and glycolysis are a typical characteristic of cancer cells. Along with prostate cancer and lymphomas, GBM belongs to the subset of tumours, in which essentially all glycolysis genes are overexpressed (Altenberg, Greulich, 2004). The glucose turnover in those proliferating cancer cells runs up to 200 times higher than in resting cells. This finding has resulted in the development of the clinical 2-(18F)-fluoro-2-deoxy-D-glucose positron emission tomography (PET) tumour imaging technique. Furthermore, proliferating cells ferment glucose into lactate even in the presence of high oxygen levels. This phenomenon was first described by the Nobel Prize winner Otto Warburg in the year 1930, and is called aerobic glycolysis or the Warburg effect

(WARBURG, 1956a, 1956b). The rate of glycolysis in GBM is several folds higher compared with normal brain, which itself utilizes 60% of a person's daily glucose intake (Basic neurochemistry, 1999). This is not surprising considering the fact that even normal glial cells of the brain have higher glycolytic rates than neurons (Oudard et al., 1996), and GBM usually has hyperactive RTK/RAS/PI3K signalling. As metabolic enzymes (e.g. IDH1, LDH-A or the pyruvate kinase M2 (PKM2)) and metabolites (e.g. 2-hydroxyglutarate or lactate) are not just simply a byproduct of cellular proliferation, but also play an important oncogenic or prognostic role in GBM (Agnihotri, Zadeh, 2016, McKinney et al., 2019), the tumour metabolism was integrated as a hallmark of cancer, that might have a crucial role in the development of treatment strategies of the GBM (Hanahan, Weinberg, 2011).

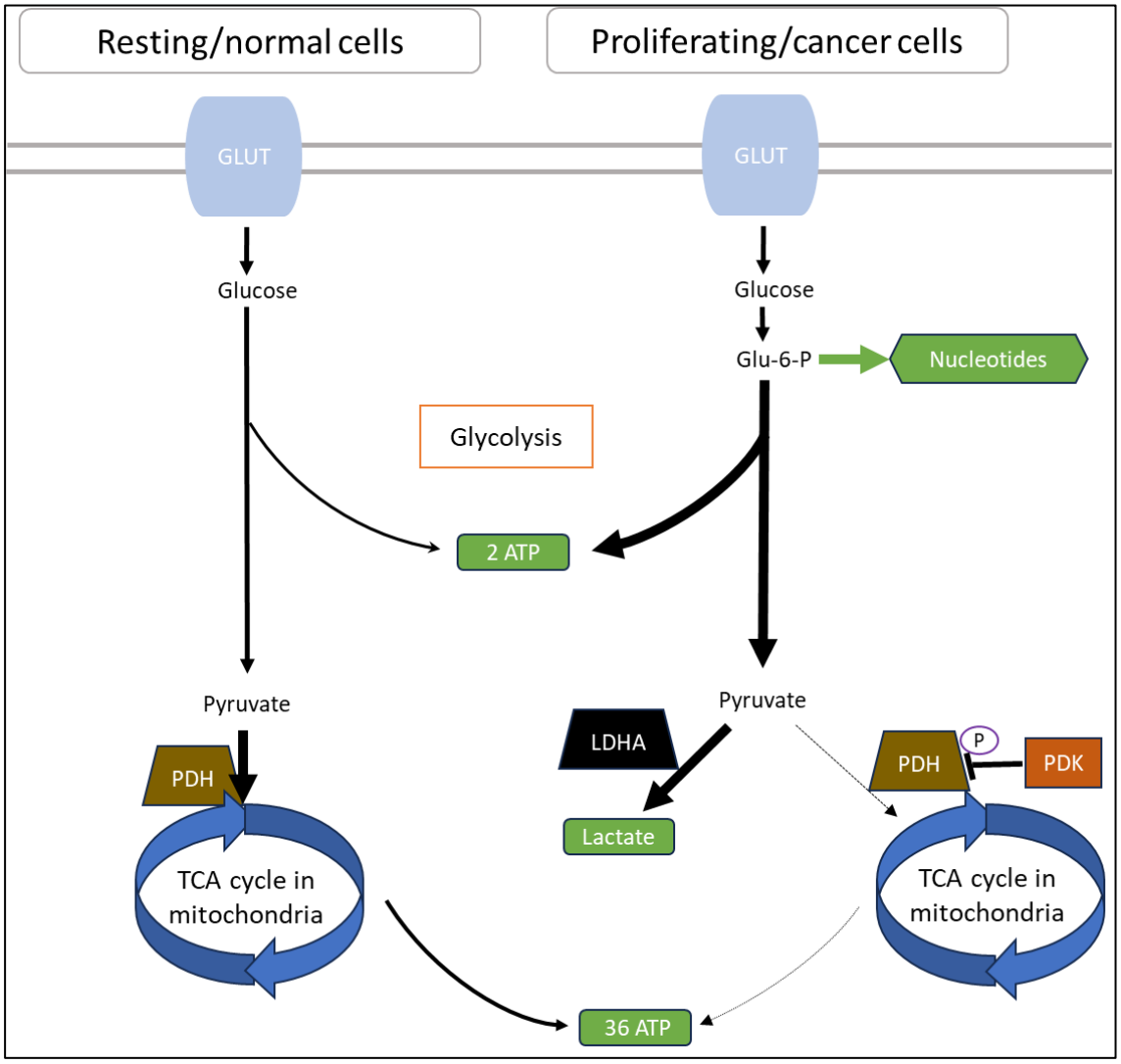


Figure 1. Graphical illustration of the Warburg effect (adapted from Agnihotri & Zadeh, 2016).

Normal metabolism: One glucose molecule trespasses the blood-brain barrier's GLUT1 and GLUT3 glucose transporters entering the cell and undergoing glycolysis, which through several enzymatic reactions converts it to one molecule of pyruvate (Patching, 2017). Pyruvate then enters the mitochondria and undergoes the tricarboxylic acid (TCA) cycle to generate a total amount of 36 adenosine triphosphates (ATP) throughout the process of oxidative phosphorylation.

Cancer Cells: Pyruvate is a critical metabolic control point. Its cellular amount is regulated by the pyruvate kinase activity of the enzyme PKM2 (not shown here). Pyruvate may either be converted to lactate by lactate dehydrogenase A (LDH-A) or imported within the mitochondria to be converted to acetyl coenzyme A (AcetylCoA) by the pyruvate dehydrogenase (PDH) to fuel the TCA cycle. Activation of the transcription of both the lactate dehydrogenase A (LDH-A) and the pyruvate dehydrogenase kinase (PDK), which phosphorylates and inactivates PDH, has been reported in GBM both through oncogenic signalling and through the hypoxia inducible factor (HIF). Although ATP production is less efficient in aerobic glycolysis compared with complete oxidative phosphorylation of glucose as in normal cells, tumour cells use aerobic glycolysis to generate precursors for anabolism to grow and generate enough ATP for cell function maintenance. By modulating glycolysis, tumour cells can divert glycolytic intermediates to generate biomass, namely nucleotides, lipids, proteins, and also NADPH to deal with oxidative stress. The cells also generate large amounts of lactate for several tumour-driving growth functions (Agnihotri, Zadeh, 2016, Garnier et al., 2019).

Adenosine triphosphate (ATP) is the energy currency of the cell as ATP hydrolysis releases energy that allows the occurrence of various essential cellular biochemical reactions. Despite the rather inefficient way of ATP generation, the Warburg effect contributes to anti-apoptosis, redox balance and tumour anabolism via increased biosynthesis of macromolecules and nucleotides for DNA synthesis (Hsu, Sabatini, 2008). Recently LDH-A levels, which are a mediator of the Warburg effect, were proposed as a new biomarker of GBM cells with high invasive potential, altered oxidative stress, and resistance to EGFR inhibition, revealing a therapeutic target whose inhibition should limit GBM invasion (McKinney et al., 2019). Moreover, elevated LDH is sufficient to drive the transition from hyperplasia to neoplasia in a *Drosophila* genetic model of EGFR-driven epithelial cancer (Eichenlaub et al., 2018). Further details on the molecular mechanisms of the Warburg effect will be given in the PKM2 chapter.

1.1.3 Intratumoural heterogeneity

During tumour development, GBM accumulates mutations and continues to evolve. This evolution generates a molecular heterogeneity within the tumour, which predisposes patients to inferior responses to all known non-invasive anticancer therapies (Dagogo-Jack, Shaw, 2018). For example, high levels of expression heterogeneity of RTKs within

individual tumours diminish the effectiveness of anti-EGFR therapy (Furnari et al., 2015). As it is common for a GBM tumour to show gene expression patterns that align with more than one of the molecular subtypes listed above, GBM displays a high level of intratumoural heterogeneity. Consequently, the tumour contains a subpopulation of cancer stem-like cells (CSCs). This small population of tumour cells can drive tumour initiation and maintenance, and even the tumour heterogeneity itself can be reconstituted with only the transplantation of CSC population expressing characteristical surface markers such as CD133 or CD15. Only the CSCs possess the self-renewal capabilities that drive the tumour growth *in vivo* (Chen et al., 2010, Soeda et al., 2015). Particularly important from the clinical point of view is their elevated resistance to chemo- and radiotherapy. Those therapies lead to an enrichment of the fraction of CSCs, which have an enhanced tumorigenicity compared to the tumour bulk (Bao et al., 2006, Li et al., 2008). This might be an explanation for the therapeutic limitations of the conventional therapies that target the tumour bulk population of neoplastic cells but may allow surviving CSCs to regenerate the tumour. The targeting of CSCs is therefore of enormous relevance for the development of novel anti-tumour therapies that have the aim to lead to complete and lasting tumour elimination. Interestingly, CSCs can be identified in most tumour types. The CSCs in GBM are typically found in both perivascular sites and hypoxic zones of the tumour, and the CSC phenotype maintenance can be driven by hypoxia (Calabrese et al., 2007, Garvalov, Acker, 2011), which constitutes another reason for intratumoural heterogeneity. Factors from the tumour microenvironment like hypoxia determine the heterogeneity of tumours, too. The intratumoural heterogeneity develops as tumour cells experience distinct selection pressures in different parts of the tumour (Furnari et al., 2015). Several intratumoural hypoxia zones are a typical feature in GBM. Cells located further from blood vessels experience decreased oxygen exposure and manifest enhanced levels of HIF-1. As HIF-1 stimulates the aerobic glycolysis, and hypoxic cells tend to consume higher levels of glucose than non-hypoxic cells, the hypoxia zones within a GBM contribute to metabolic heterogeneity within the tumour, too (Kim, DeBerardinis, 2019, Sonveaux et al., 2008). This might be clinically relevant, as heterogeneity of hypoxia and glucose uptake predicts poor outcomes in human solid tumours (Kim, DeBerardinis, 2019, Vaupel, Mayer, 2007).

1.2 EGFR signalling

EGFR is frequently overexpressed in a variety of malignant tumours such as head & neck, breast, renal, lung, colon, prostate and ovarian carcinomas (Grandis, Sok, 2004). It is the best studied transmembrane glycoprotein of the EGF tyrosine kinase receptor family consisting of EGFR (ErbB1), Her2 (ErbB2), ErbB3 and ErB4 (Wang, 2017). The EGFR gene is located on chromosome 7 (Kondo, Shimizu, 1983), and the molecular weight of this receptor is 170 kDa. EGFR consists of extracellular, transmembrane, intracellular and cytoplasmic domains. Its kinase domain is located intracellularly. EGFR forms either a homodimer or a heterodimer with other members of the EGF tyrosine receptor family (Endres et al., 2011), and its activation process is illustrated in Figure 2.

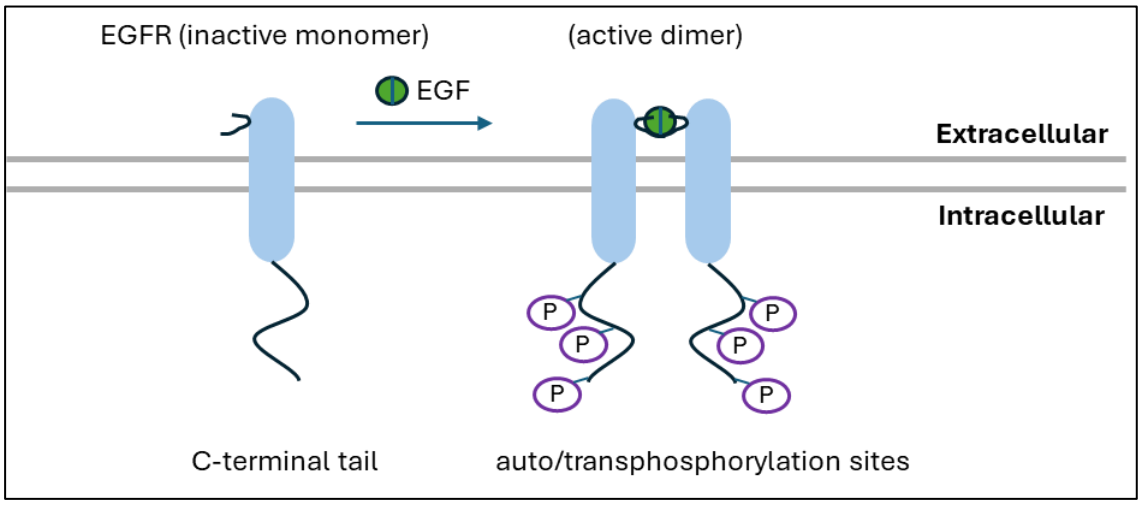


Figure 2. Activation of EGFR (adapted from Endres et al., 2011). If growth factors such as EGF bind to EGFR's extracellular domain, the structure of the extracellular domain changes, triggering dimerization of the intracellular domain and the activation of the intracellular kinase domain. Subsequently, EGFR autophosphorylates, transphosphorylates and regulates several phosphorylation-driven RTK signalling pathways such as the ERK (extracellular-signal regulated kinase), Akt (protein kinase B) and Stat (signal transducer and activator of transcription) pathways (Hubbard, Miller, 2007).

The activation of the EGFR signalling pathways, and especially of the Akt (= protein kinase B) and ERK (extracellular-signal regulated kinase) pathways, is linked to various cancers. It is worth noting that various other proteins like FGFR also may activate the EGFR downstream mediators. Both the EGFR-mediated cytoplasmic and the nuclear signalling is involved in many biological processes and cellular functions, e.g. embryonic development, chromatin remodelling, DNA-repair, apoptosis, cell-cycle, motility, differentiation, growth, tumorigenesis and metastasis (Wee, Wang, 2017). Overexpressed

and overactivated EGFR receptors are markers of poor prognosis in GBM patients. Furthermore, constitutive co-activation of EGFR-Ras and the Akt pathway in the glia of *Drosophila* was sufficient to inducing invasive GBM-like cell formation, implicating an important role in GBM tumorigenesis (Read et al., 2009). EGFR alterations including EGFR mutations, rearrangements and focal amplifications occur in total in about 57% of the GBMs and are by far the most dominant RTK lesion in GBM (Brennan et al., 2013). Moreover, EGFR alterations are usually the sole RTK lesions in GBM (Furnari et al., 2015). The most common EGFR mutant that is found in 25% of the GBMs is the constitutively active EGFR variant (v) III. It lacks the extracellular domain from exons 2 to 7 (out of 16), doesn´t internalize upon activation and preferentially signals through the Akt signalling pathway (Huang et al., 2009, Humphrey et al., 1988).

Accordingly, the inhibition of EGFR pathways is a method successfully used in anti-cancer treatment regiments, such as lung or colon cancer treatment, but so far not successfully in GBM. Mutations of EGFR in cancer cells that lead to a constitutive gain-of-function phosphorylation of the protein may turn cancer cells vulnerable to ionizing radiation and/or a receptor kinase inhibitor like erlotinib. It seems that those cells may become addicted to this signalling pathway (Weinstein, Joe, 2006). The inhibitors currently used can be divided into antibodies that target EGFRs extracellular domain (e.g. Cetuximab), and small molecule inhibitors that target its intracellular ATP-binding domain (e.g. erlotinib or gefitinib). EGFR signalling pathways and inhibitors are illustrated in Figure 3.

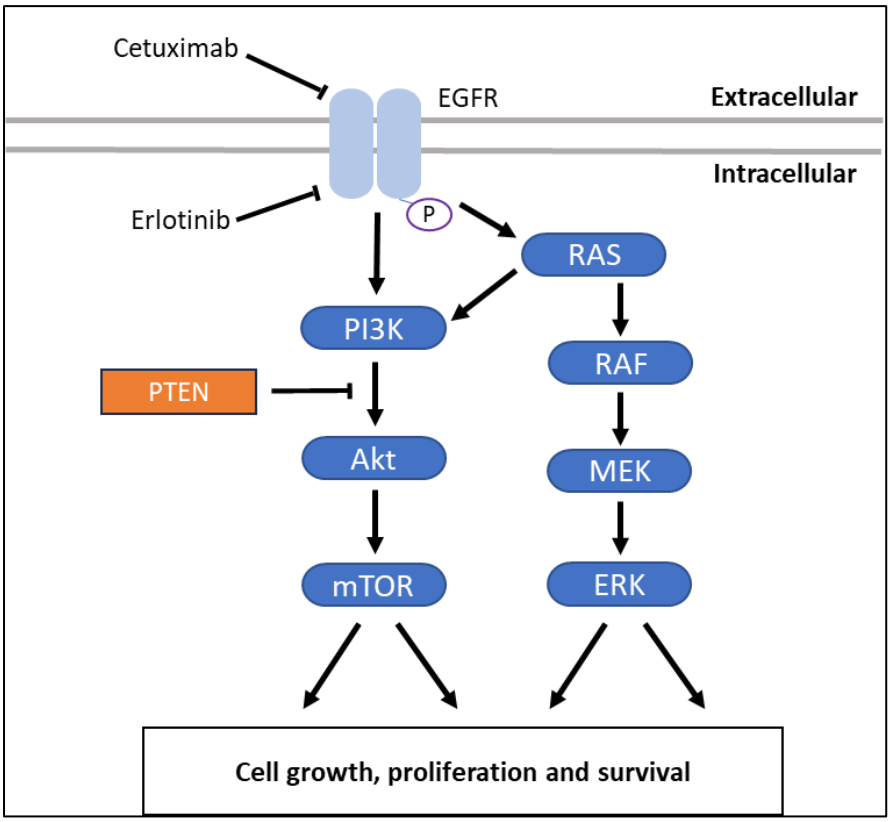


Figure 3. The most relevant nodes of the complex EGFR signalling network (adapted from Dienstmann et al., 2011). In total, the whole signalling network contains at least 211 reactions involving 322 components (Oda et al., 2005). Activated Akt and ERK signalling pathways may induce cancer cell proliferation and invasion. In addition, activated Akt is inducing cancer stem cell renewal and differentiation (Cheng et al., 2013). The Akt signalling may be activated independent of EGFR through genetic aberrations such as the deletion of PTEN or a PI3K mutation. Anti-EGFR targeted monoclonal antibodies such as Cetuximab or small molecule inhibitors like erlotinib may bind to EGFR and block its function (Dienstmann et al., 2011).

Besides regulating ERK, Akt, and Stat signalling pathways, EGFR also displays other functions that have been shown in recent years. Activation of EGFR in GBM cancer cell lines promotes the Warburg effect by increasing glucose uptake and lactate production in a PKM2-dependent manner. As such, EGFR-induced NF-kB activation was demonstrated to upregulate PKM2 expression and promote tumorigenesis (Yang et al., 2012a). The activation of EGFR induces the translocation PKM2 into the nucleus, where it induces expression of oncogenic genes essential for the Warburg effect, such as c-Myc (Yang et al., 2011). C-Myc is a transcription factor which induces pluripotency *in vitro* and has cancer and Warburg effect promoting properties (Stine et al., 2015, Takahashi, Yamanaka, 2016). Furthermore, EGFR trafficking to specific cellular locations like the mitochondria or the nucleus corresponds to treatment resistance and abnormal cell growth

(Che et al., 2015). It was reported that EGFR itself may translocate to the nucleus, where it can form complexes with transcription factors such as Stat3, regulating gene transcription and resistance towards targeted anti-cancer therapy (Li et al., 2009, Lo et al., 2005). As shown recently, nuclear EGFR and PKM2 form a complex that may even regulate the cancer stem-like phenotype through the transcription of CSC-related genes, suggesting a wider role of nuclear EGFR in tumour resistance than initially thought (Shi et al., 2018).

1.2.1 Regulation

The EGFR signalling may be regulated by various mechanisms. These include EGFR endocytosis, lysosomal degradation, ubiquitin-proteasome dependent degradation, and nuclear shuttling (Alwan et al., 2003, Lo et al., 2006, Ravid et al., 2004, Sebastian et al., 2006, Wiley, Burke, 2001). In addition, EGFR function may be controlled by various posttranslational modifications (PTM) such as glycosylation, neddylation, sumoylation, ubiquitination, phosphorylation and lysine acetylation (Malik et al., 2019). Acetylation at certain lysine sites of EGFR is important for the regulation of EGFR endocytosis (Goh et al., 2010). Moreover, the deacetylase inhibitors trichostatin A (TSA) and SAHA increase both EGFRs acetylation and phosphorylation (Song et al., 2011). Phosphorylation is the best known posttranslational regulatory mechanism of EGFR. The intracellular domain of EGFR harbours several critical tyrosine phosphorylation sites such as Y845, Y992, Y1045, Y1068 and Y1173, which in turn regulate different signalling pathways (Sordella et al., 2004). Y845 is associated with Stat signalling, Y1045 with EGFR ubiquitination and degradation through Cbl-interaction (Sorkin, Goh, 2008), while other sites like Y1068 are linked to the ERK and Akt signalling pathways (Sordella et al., 2004). The regulation mechanisms of the individual signalling sites remain largely unknown. Besides EGFR ligands, many proteins interact with EGFR. As proteomic analysis revealed, over one hundred proteins interact with EGFRs cytoplasmic tail, binding to about twenty intracellular phosphotyrosine residues (Morandell et al., 2008). EGFRs phosphorylation status is negatively regulated by several phosphatases (Tiganis, 2002), and the deregulation of phosphatases may cause cancers and other diseases (Ostman et al., 2006). The inhibition of phosphatases promotes general activation of RTKs in cells (Östman, 2001). Continual EGFR signalling activates the NADPH-oxidase, which induces the production of various instable reactive oxygen species (ROS) (Bae et al., 1997). These ROS inhibit the activity of the phosphatases by oxidizing their cysteine site, leading to

enhanced EGFR signalling in form of a transient positive feedback mechanism (Tonks, 2006). On the other hand, neddylation and Cbl-mediated ubiquitination downregulates EGFR signalling (Sorkin, Goh, 2008). Ubiquitination modulates EGFRs recycling, translocation and proteasome degradation, and it has been shown that the function of EGFR may be regulated by mono- , multi-, and poly-ubiquitination (Huang et al., 2006, Mosesson et al., 2009).

Besides EGFR signalling, its trafficking is also extensively regulated by protein-protein interactions (Tong et al., 2014). After the activation of EGFR, it is internalized and may be transported to the endosomes or to the nucleus within less than 15 min (Wang et al., 2010a). From the endosomes, where EGFR may remain for about 1 hour, EGFR is either degraded, or recycled back to the cell surface (Sebastian et al., 2006, Wang et al., 2010b). Evidence suggests that activated EGFR may continuously signal while being translocated (Wang et al., 2002). The EGFR shuttling process can also be regulated indirectly. For example, the hypoxia regulated prolyl hydroxylase PHD3 downregulates EGFR signalling by binding to EGFRs endocytic adaptor Eps15 in GBM cell lines (Garvalov et al., 2014). Another indirect regulator is the histone deacetylase 6 (HDAC6), which can regulate the turnover of EGFR by controlling alpha-tubulin acetylation and thus receptor trafficking along microtubules (Deribe et al., 2009).

Besides EGFR-dependent regulatory mechanisms the downstream signalling is also being regulated by multiple other ways. There are several posttranslational modifications such as phosphorylation, ubiquitylation or acetylation that regulate the downstream mediators of EGFR signalling, and there are diverse mutations that may constitutively activate its pathways, such as the PTEN mutation. An essential problem of cancer treatment therapies is the development of resistance against the treatment regimen through such mutations. EGFR alterations frequently co-occur with mutations of PI3K and PTEN deletions, which activate the Akt signalling pathway independently of EGFR (Brennan et al., 2013). Importantly, several EGFR downstream mediators are being regulated by PKM2, which will be discussed below.

1.2.2 EGFR is a potential therapeutic target in GBM

The most used drugs inhibiting EGFR signalling are the tyrosine kinase inhibitors (TKIs). There are first-generation TKIs like erlotinib which are small molecules competing for the ATP-binding site in the TK domain of EGFR, which thus abolish the phosphorylation

and downstream signalling of EGFR. Erlotinib is orally active and, for GBM patients, constitutes the best explored EGFR inhibitor in clinic (Zhao et al., 2015). As drug, erlotinib is currently used for treatment of locally advanced or metastatic non-small cell lung cancer (NSCLC) and pancreatic cancer. As part of the World Health Organization's List of Essential Medicines, erlotinib is effective in patients both with or without EGFR mutations in NSCLC, but shows best effectiveness with EGFR mutant cancers (Kobayashi, Hagiwara, 2013). For this reason, erlotinib is mainly used in patients with either an EGFR exon 19 deletion (del19) or exon 21 (L858R) substitution mutation. After adding it to chemotherapy, it improves overall survival by 19%, and enhances the progression-free survival by 29% compared to chemotherapy alone (Cappuzzo et al., 2010). As with other TKI inhibitors patients regularly develop resistance. For erlotinib, this mainly occurs 8–12 months after treatment initiation. Over 50% of resistance development is caused by the T790M-point-mutation in the ATP binding pocket of the EGFR kinase domain (Balak et al., 2006). This resistance mechanism may be overcome by use of third-generation TKIs like osimertinib which target the T790M mutation. Unfortunately, the tumour becomes resistant even to those drugs after approximately 10 months of treatment (Patel et al., 2017). The second way by which the NSCLC tumour develops resistance, is the amplification of the tyrosine kinase MET, occurring in 20% of the cases. MET then leads to ErbB3-dependent activation of Akt signalling (Bean et al., 2007).

Despite the frequent prevalence of wild-type and altered EGFR in GBM, none of the current TKIs has achieved any therapeutic benefit for GBM patients in clinical trials. However, one study showed that treatment with the combination of erlotinib, temozolomide and radiotherapy significantly prolongs survival in the subgroup of PTEN and MGMT promoter methylation positive GBM patients (Prados et al., 2009). Yet, most studies show no significant survival benefit (Le Rhun et al., 2019). There may be several reasons for this. According to one theory, the heterogeneity of cancer cells in GBM leads to very rapid resistance towards particular therapeutic reagents (Thorne et al., 2016). Although there are some EGFR mutations or deletions that make the cancer cells more sensitive towards TKI like gefitinib (Sordella et al., 2004), somatic mutations and deletions of EGFR also play a critical role in the development of resistance towards TKI in EGFR-expressing cancer cells (Linardou et al., 2009). Moreover, some of the mutants including the extracellularly truncated 145 kDa mutant EGFRvIII lead to resistance

towards both radio- and chemotherapy in GBM (Mukherjee et al., 2009, Tanaka et al., 2011). The crosstalk between wild-type EGFR and EGFRvIII promotes tumorigenesis, and often, different EGFR alterations coexist within the same tumour (Furnari et al., 2015).

Other factors leading to resistance development are EGFR-encoding small circular extrachromosomal DNA elements called double-minute chromosomes, which may dynamically become downregulated by TKIs. Upon drug withdrawal they can reappear and restore EGFR signalling (Nathanson et al., 2014). Furthermore, the co-occurrence of different RTK alterations expands resistance towards specific RTK inhibitors as well. For example, in some cases of NSCLC resistance development the alterations in EGFR signalling come along with concomitant alterations in other RTKs like MET, driving resistance towards EGFR inhibitors (Stommel et al., 2007).

For those reasons, clinical applications of the EGFR inhibitors might be limited or only effective to a specific subgroup of patients over a relatively short period of time. Yet, combinational approaches that target heterogeneous tumour populations were successful in preclinical studies. The combination strategy has been suggested as a promising method not only to overcome resistance but also to reduce the dosages of both agents to minimise adverse effects and enhance the anti-cancer effect of individual strategies (Yang, Tam, 2018). For example, the combination therapy of TKI with deacetylase inhibitors such as SAHA or trichostatin A (TSA) has been proposed for treating cancers with aberrant expression of EGFR. Although the use of TSA in EGFR-expressing tumour cells treatment enhanced EGFR phosphorylation, evidence from pre-clinical and clinical trials on combination therapy demonstrated significant enhancement of therapeutic efficacy (Zhou et al., 2006). All in all, the therapeutic strategy of targeting various oncogenic stimuli simultaneously is proving to be effective and seems like a trend for the future. Considering EGFR targeting, potential future therapeutic strategies include the targeting of multiple RTKs, EGFR mutants or mediators of the EGFR signalling pathway at the same time (Le Rhun et al., 2019, Thorne et al., 2016).

1.3 Pyruvate kinase M2

PKM2 is one of the four pyruvate kinase (PK) isoforms in mammals that catalyse the conversion of phosphoenolpyruvate (PEP) and ADP to pyruvate and ATP in the last

irreversible step of glycolysis. The four PK isoforms PKR, PKL, PKM1 and PKM2 are transcripts of two distinct genes. The gene PKLR encodes for PKL and PKR. PKL is found mainly in the liver and the kidneys, whereas PKR is expressed in red blood cells. PKM1 and PKM2 are alternative splice variants encoded by the PKM gene. While PKM1 contains exon 9, PKM2 instead contains the exon 10 (Wong et al., 2015). Metabolic hormones like insulin or triiodthyronine-T3 regulate the PKM gene expression. Hypoxia, glucose stimulation, and several signalling pathways, including RTKs like EGFR stimulate the PKM gene transcription (Prakasam et al., 2018). Intriguingly, PKM2 may interact with HIF-1a, further enhancing HIF-1a mediated PKM gene transcription (Luo et al., 2011a, Prigione et al., 2014, Yang et al., 2012a). The RTK/Akt pathway induces mTOR signalling, which simultaneously activates HIF-1a-mediated PKM transcription and c-Myc-dependent pre-mRNA splicing, thus increasing the production of PKM2 mRNA (Sun et al., 2011).

While the PKM1 isoform is predominantly expressed in brain, heart and skeletal muscle tissues, PKM2 is mainly expressed in fat tissue and highly proliferative tissues, e.g. in embryonic and cancer tissues (Dayton et al., 2016). High levels of PKM2 in the blood of cancer patients are being found in cancers of the gastrointestinal tract, the kidneys, the lung and the ovaries. The blood levels of dimeric PKM2 also correlate with tumour progression, and there is evidence that it stimulates endothelial cell migration, facilitating tumour angiogenesis (Ahmed et al., 2007, Hardt et al., 2000, Kumar et al., 2007, Li et al., 2014a, Schneider, 2006, Tonus et al., 2012, Ugurel et al., 2005, Weinberger et al., 2007).

During the cancer formation, the expression of PKM2 increases while the expression of the respective PKM1/L/R decreases (He et al., 2017, Mazurek, 2011). Nonsense and missense mutations in PKM2 do occur in human tumours (Gupta et al., 2010, Iqbal et al., 2014). This is consistent with the idea that tumours select for low PK activity, supporting the hypothesis that regulation of PKM2 glycolytic activity drives selection for the PKM2 isoform in tumours (Israelsen et al., 2013). The significance of PKM2 regulation for the cellular glucose metabolism is well known. When glucose concentration is low, tumour cells use the limited glucose for mitochondrial respiration to generate ATP for survival. When glucose concentration is high, tumour cells decrease PKM2 activity in order to transform glycolytic metabolites into biomass (Li et al., 2014b). Conversely, high PK activity due to PKM1 overexpression or pharmacological activation of PKM2 may slow tumour growth by decreasing the levels of metabolites that are important for biosynthesis

in vivo (Anastasiou et al., 2012). For example, PKM2 knockdown decreases glutathione levels, leading to increased ROS vulnerability in cancer cells. By contrast, an increased AMP:ATP ratio due to PKM2 knockdown increases levels of AMPK. In turn, the increase of AMPK induces apoptosis in several cell lines including glioma (Mukherjee et al., 2008). In GBM the PKM1-PKM2 isoform switch does occur (He et al., 2017). While lower grade gliomas all have moderately increased levels of PKM2 protein expression relative to normal brain, GBMs shows a 3-5 fold increase in PKM2 mRNA and protein expression (Mukherjee et al., 2013). The low levels of PKM1 expression are important for GBM cell growth as PKM1 overexpression inhibits the growth of GBM cells. In contrast to normal brain tissue, the survival of GBM stem cells and cell lines is dependent on PKM2 expression (Kefas et al., 2010).

The tumour growth promoting effect of PKM2 is astounding, as the translated PKM2 differs from PKM1 only by a region counting 23 amino acids, that does not contribute to its pyruvate kinase activity (Noguchi et al., 1986). However, the according region harbours sites important for the stability of the tetramer formation (Dombrauckas et al., 2005). While PKM1 forms a stable and constitutively active tetramer, the stability of the PKM2 tetramer formation can be affected by numerous compounds and posttranslational modifications. For this reason, distribution of total PKM2 ranges from tetramers to dimers and monomers. Some of the compounds and posttranslational modifications which destabilize the PKM2 tetramer act as nutrient sensors that promote the Warburg-effect (Lv et al., 2013). Unsurprisingly, the dimer-tetramer ratio is a very important factor for regulating cell proliferation and survival (Lv et al., 2011).

The dimer-tetramer ratio is being regulated by various environmental factors, oncogenes, tumour suppressors and metabolites, and the transversion from tetrameric to dimeric PKM2 plays an important role in tumour cell energy supply, cell dedifferentiation, invasion, resistance, metastasis and cell proliferation. Interestingly, the PKM2 dimer lacks PK activity, displaying on the other hand various oncogenic functions such as a protein kinase activity and the ability to translocate to the nucleus and regulate gene expression (Zhang et al., 2019). In all cases, the non-PK functions are found only with PKM2, and not with PKM1, suggesting that some of those drive PKM2 selection in cancer. In Figure 4 both the regulatory mechanisms of PKM2 and its nuclear and glycolytic functions, which will be elucidated in the later chapters, are summarised in a simplified manner.

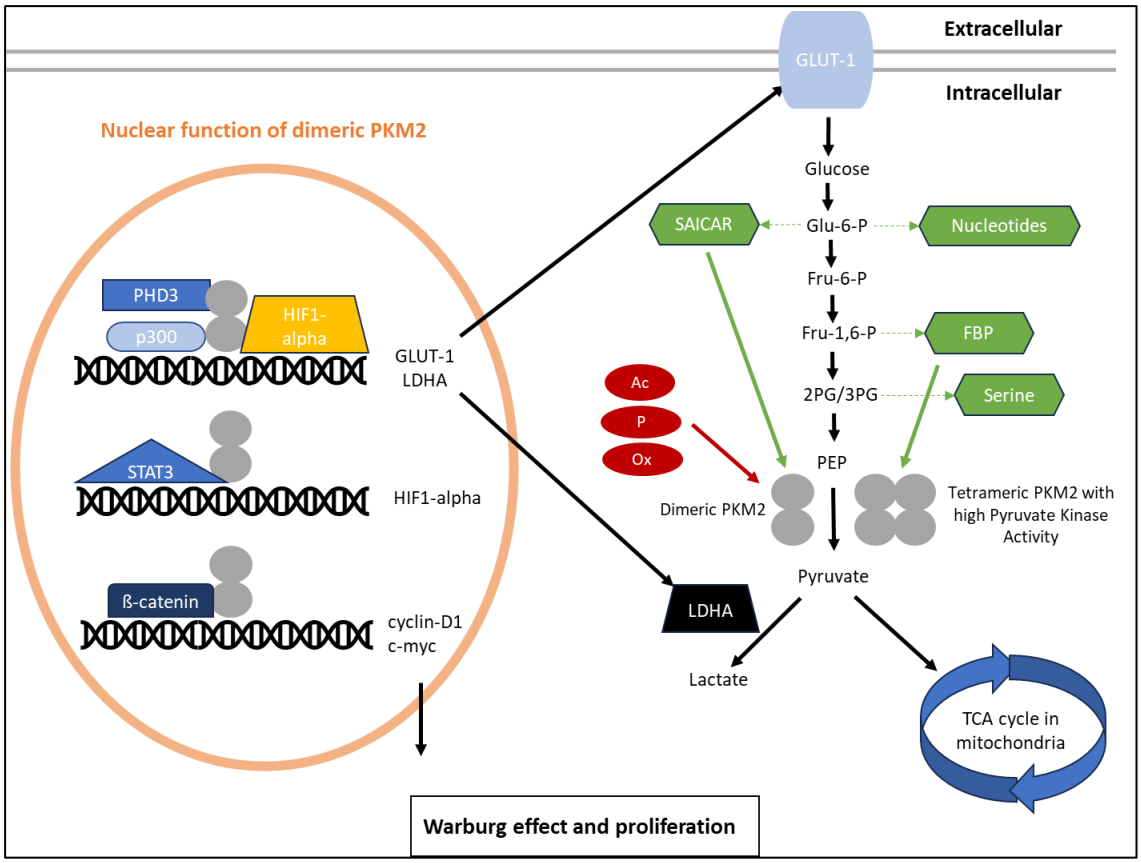


Figure 4. Regulation, nuclear functions and the cytosolic glycolytic functions of PKM2 (adapted from Alves-Filho, Palsson-McDermott, 2016). The dimer-tetramer ratio of PKM2 regulates the Warburg effect, as the PKM2 tetramer has high pyruvate kinase activity and represents a critical checkpoint in metabolism. A tetramer activator function for fructose-1,6-biphosphate (FBP) is indicated. The PKM2 dimer is able to translocate into the nucleus and regulate gene transcription of pro-glycolytic and tumour-driving genes together with HIF-1α, STAT3 or ß-catenin. An important activator of pyruvate and protein kinase activity of the PKM2 dimer is phosphoribosylaminoimidazolesuccinocarboxamide (SAICAR), an intermediate of the pentose phosphate pathway. Furthermore, PKM2-dimer function is regulated through posttranslational modifications like acetylation, phosphorylation or oxidation (Demaria, Poli, 2012, Yan et al., 2016). See text for further details.

1.3.1 Regulation of glycolytic functions of PKM2

PKM2 tetramers are constitutively active and are retained in the cytosol (Mazurek et al., 2005). The PKM2 tetramer function is being regulated by a variety of compounds and signalling pathways. Relevant tetramer activators are fructose-1,6-biphosphate (FBP), the upstream intermediate of glycolysis, and the amino acids serine, phenylalanine and alanine. Those compounds are related to the cellular availability of glucose and metabolic intermediates (Zhang et al., 2019). FBP and serine both can allosterically activate the PKM2 tetramer by causing a reversible dimer-to-tetramer conversion (Ashizawa et al.,

1991, Chaneton et al., 2012, Dombrauckas et al., 2005). The binding of FBP causes PKM2 to adopt a stable, active conformation similar to that of PKM1 (Anastasiou et al., 2012). Consequently, less glycolytic intermediates are being produced, as the glycolytic intermediate PEP is being converted to pyruvate until FBP levels drop below a critical value that allow the PKM2 tetramer to dissociate to the dimeric form. A similar regulation takes place with the amino acid serine. In the case that the serine is abundantly available inside the cell, PKM2s enhanced PK activity does emerge. However, when serine availability is limited, the inhibition of PK activity enables the accumulation of glycolytic intermediates essential for serine biosynthesis, which enables growth and proliferation (Chaneton et al., 2012).

The PK activity of PKM2 may also become reduced via various PTMs, which are being enhanced in various types of tumours (Prakasam et al., 2018). PTMs at the intersubunit interface region of PKM2, namely acetylation, succinylation or O-GlcNAcylation, may function as nutrient sensors as they destabilize its tetramer formation (Wang et al., 2017b). Especially phosphorylation at lysine 367 is important for PK activity. The mutant PKM2 K367M was generated in order to create a PK-inactive mutant (Yang et al., 2011). Another PTM regulating the activity of PKM2 is the ROS-mediated oxidation of the cysteine 358 of PKM2, which also inhibits its PK activity. Anastasiou et al. (2011) showed that this regulation stimulates the flux of glycolytic intermediates into the pentose phosphate pathway, increasing production of antioxidants like glutathione.

Furthermore, several cancer-promoting RTKs such as EGFR, FGFR1, BCR-ABL, JAK2 and FLT3-ITD also regulate the PK activity of PKM2 as their signalling pathways promote the phosphorylation of tyrosine residues. Both the binding of phosphotyrosine peptides to PKM2 (Christofk et al., 2008b, Mason et al., 2004) and Y105 phosphorylation (Hitosugi et al., 2009), can override the FBP-binding of PKM2 and thus its tetramer formation (Gao et al., 2013).

Last but not least, several proteins including the CSC marker CD44 also interact with PKM2, and also increase the PKM2 phosphorylation at threonine 105 residue. Thus, CD44 ablation can rewire aerobic glycolysis into mitochondrial respiration, which significantly enhances sensitivity to cisplatin in a colorectal xenograft tumour model (Tamada et al., 2012).

1.3.2 Functional diversity of the PKM2 dimer

As mentioned above, there are many ways such as PTMs and protein interactions with e.g. phosphotyrosine residues that induce PKM2 dimer formation. Several proteins that bind to the intersubunit interface region of PKM2 may promote PKM2 dimerization, subsequently regulating the Warburg effect and stimulating the translocation of PKM2 to the nucleus which regulates gene expression (Wang et al., 2014). Besides this, PKM2 protein interactions can also regulate cell growth via other mechanisms. For example, PKM2 may bind with HuR, an RNA binding protein which plays an important role in the control of mRNA stability and translational efficiency, to promote cell cycle progression and proliferation of glioma cells (Mukherjee et al., 2016).

Interestingly, the levels of nuclear PKM2 correlate with cell proliferation. One explanation is that nuclear PKM2 promotes HIF-1-mediated tumour growth. Hypoxia promotes apoptosis in both normal and tumour cells. However, HIF-1 levels are increased in many cancers including GBM. HIF-1 plays a crucial role in initiating angiogenesis and regulating cellular metabolism to overcome hypoxia through promoting the expression of its target genes which encode for several glycolytic enzymes and the vascular endothelial growth factor (VEGF) (Vaupel, Mayer, 2007, Wheaton, Chandel, 2011, Zhong et al., 1999). A study has shown that the PHD3-hydroxylated PKM2 dimer may localize to the nucleus, where it is recruited by HIF-1 and the acetyltransferase p300 to specific regions in the DNA, leading to histone H3K9 acetylation. This promotes the transactivation of cancer cell genes encoding glucose transporters and glycolytic enzymes, e.g. GLUT1 and LDHA (Luo et al., 2011a).

Nuclear PKM2 has further significance in the process of regulating gene expression. E.g., it enhances Oct-4-mediated gene transcription (Morfouace et al., 2014). The transcription factor Oct-4 is used as a marker of cell-stemness and is sufficient to induce pluripotency in mouse adult neural stem cells (Kim et al., 2009). Furthermore, there is evidence that the PKM2 dimer may act as a protein kinase. An unbiased quantitative phosphoproteomics survey identified 974 PKM2 substrates in the proteome of renal cell carcinoma (He et al., 2016). Additionally, *in vitro* phosphorylation assays have shown that the PKM2 dimer may act as a protein kinase using PEP as the phosphate donor. An important regulator of PKM2s protein kinase activity is phosphoribosylaminoimidazolesuccinocarboxamide (SAICAR), an intermediate of the pentose phosphate pathway. Protein microarray experiments displayed more than 100

human proteins, most of them protein kinases, that are potential phosphorylation targets of PKM2-SAICAR. These potential targets include EGFR. Interestingly, PKM2-Q393K point mutation abolishes the SAICAR binding, inhibiting the protein kinase and maintaining the PK activity of PKM2 (Keller et al., 2012, Keller et al., 2014). Inside the cell, the dimeric PKM2 localizes both in the cytoplasm and in the nucleus. It binds to and stabilizes the mitochondrial outer membrane, and is found inside the endoplasmatic reticulum (ER) where it may inhibit ER stress (Liang et al., 2017, Zhang et al., 2019). Cellular compartment analysis by WoLF PSORT (https://wolfpsort.hgc.jp/aboutWoLF_PSORT.html.en) revealed that the PKM2 protein kinase substrates are mostly nuclear proteins (67,5%), followed by proteins in the cytosol (16,4%) and mitochondrial proteins (4,5%) (He et al., 2016).

PKM2 might regulate different signalling pathways with its protein kinase activity. For instance, there is evidence that PKM2 directly phosphorylates the oncoprotein Stat3, and might be the reason for its constitutive activation in tumour cells (Gao et al., 2012). The nuclear PKM2 also phosphorylates Bub3 (Jiang et al., 2014a) and histone H3, all of which may enhance GBM tumour growth. Through the phosphorylation of H3 PKM2 is also involved in epigenetic regulations of gene expressions, as the phosphorylation by the PKM2 protein kinase is required for the dissociation of HDAC3 from CCND1 and Myc promoter regions and the following acetylation of histone H3 at K9 (Yang et al., 2012b).

Another example of the protein kinase activity of PKM2 is that it was shown that AuroraB-phosphorylated PKM2 can phosphorylate MLC2, contributing to cytokinesis, cell proliferation and brain tumour development (Jiang et al., 2014b).

Besides that, dimeric PKM2 also contributes to exosome secretion. Extracellular vesicles including exosomes released from cancer cells promote tumour growth (Martins et al., 2013, McAllister, Weinberg, 2014). Phosphorylated dimeric PKM2 promotes exosome secretion of cancer cells by direct phosphorylation of SNAP-23, a protein that allows formation of the SNARE complex and subsequent exosome release (Wei et al., 2017). Those exosomes in turn may contain dimeric PKM2 (Wan et al., 2019). As extracellular dimeric PKM2 may stimulate cancer cell proliferation by activating the EGFR signalling pathway in an autocrine manner (Hsu et al., 2016), PKM2 may also be able to induce tumour growth this way. However, this is not the only disease-driving function of dimeric PKM2 outside the cancer cells. The PKM2 dimer might also regulate the cancer-associated inflammatory process. Interestingly, lactate efflux associated with the

Warburg effect attracts immune cells such as macrophages, which secrete cytokines and growth factors that drive both tumour growth and metastasis (Doherty, Cleveland, 2013). The dimerized form of PKM2 plays an important role in this process, as PKM2-dependent glycolytic reprogramming is a critical determinant of macrophage activation (Palsson-McDermott et al., 2015). E.g., SIRT5-regulated hypersuccinylation at PKM2 lysine 311 promotes its tetramer-dimer transition, which in turn enables macrophages an increased production of cytokines such as IL-1ß (Wang et al., 2017a).

1.3.3 Lysine acetylation and its impact on PKM2 and tumours

Lysine acetylation inhibits enzymatic activity of many metabolic and glycolytic enzymes by blocking their catalytic pockets. As those acetylation sites including the 433 site of PKM2 are evolutionary conserved, the theory that lysine acetylation acts as a mechanism to limit metabolism of overabundant carbon has been proposed (Nakayasu et al., 2017, Zhao et al., 2010). Acetylation is a crucial PTM of PKM2 that stimulates biosynthesis. It is regulated by acetyltransferases and deacetylases. The process is illustrated in Figure 5.

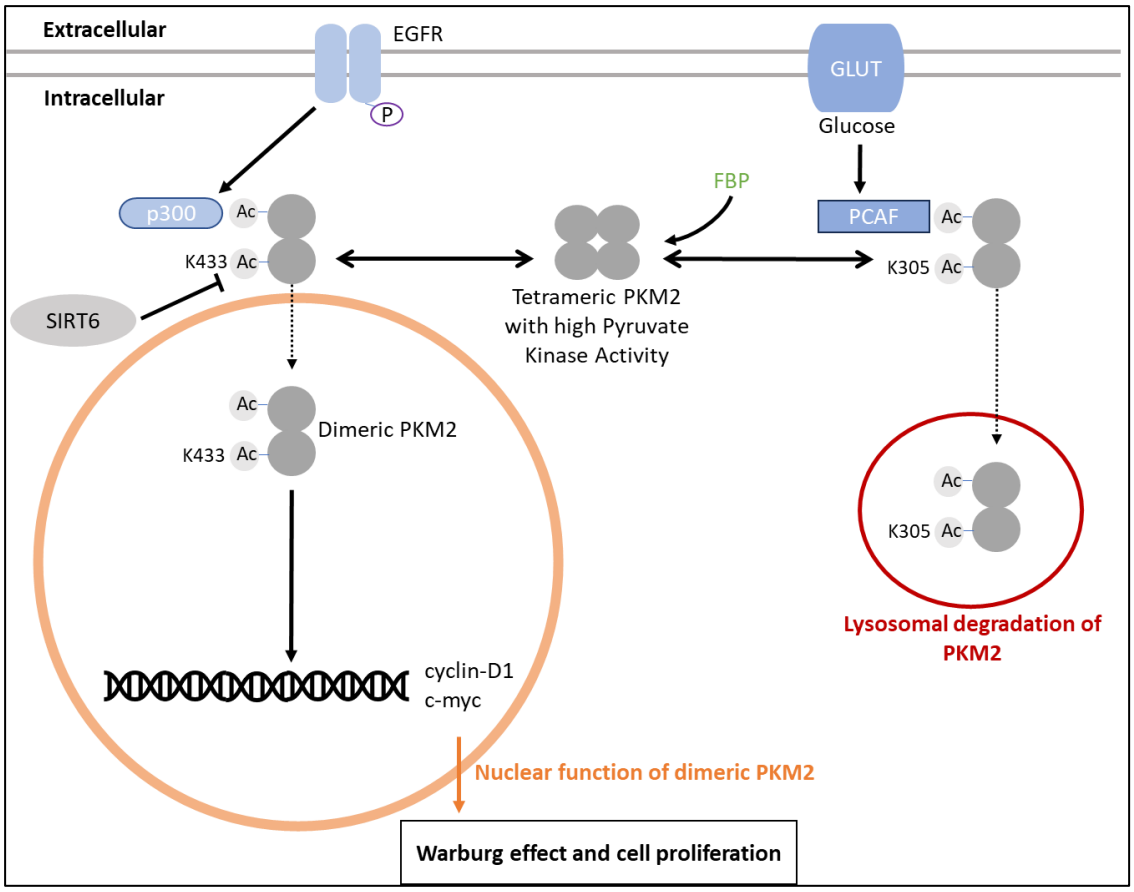


Figure 5. Regulation of PKM2 by lysine acetylation (adapted from Prakasam et al., 2018). The acetylation of PKM2 at lysine 433 (K433) is upregulated by EGF stimulation in a p300-dependent manner and downregulated by the lysine deacetylase SIRT6. Acetylation at K433 disrupts the binding with FBP, enhances the nuclear translocation of PKM2 dimers and promotes cell proliferation. Acetylation at K305 by the acetyltransferase PCAF on the other hand increases glucose-induced PKM2-degradation as it stimulates the lysosomal uptake of PKM2 (Prakasam et al., 2018).

Lysine 305 and 433 are both important acetylation sites of PKM2. The acetylation on lysine 305 (K305) gets stimulated by high glucose concentration. Mutation of K305 reduced the PKM2 acetylation by 45%. PKM2 binds to the acetyltransferase PCAF which acetylates K305. The acetylation at K305 leads to glucose-induced PKM2-degradation through increased uptake by lysosomes through chaperone-mediated autophagy. Lysine-substitution with the acetylation-mimic glutamine (K305Q) promotes the accumulation of glycolytic intermediates and the tumour growth both *in vitro* and *in vivo* (Lv et al., 2011). Park et al. (2016) found that the acetylation of site 305 is being negatively regulated by the deacetylase sirtuin 2 (SIRT2).

Lysine 433 is encoded by the M2-specific exon 10, being conserved among all vertebrate PKM2. The K433 site is important for phosphotyrosine-binding (Christofk et al., 2008b),

and its acetylation is enriched in cancer cells. The acetyltransferase p300 acetylates PKM2 at K433 *in vitro* and knockdown of p300 diminishes acetylation at K433. Furthermore, it has been shown that the oncoprotein E7 from the high-risk human papilloma virus 16 (HPV 16) promotes the binding of PKM2 to p300. The K433Q point mutation disrupts the binding with FBP. K433 site gets acetylated upon mitogenic and oncogenic signalling pathways by p300, promoting PKM2s dimeric nuclear protein kinase activity (Lv et al., 2013, Zhang et al., 2019). The acetylation levels at this site rise by cell cycle stimulation, the oncoprotein E7 from the HPV16 and EGF, while they drop in starvation conditions and through intercellular contact (Lv et al., 2013). The acetylation status of PKM2s lysine 433 is enhanced after treatment with the deacetylase inhibitor mixture of nicotinamide (NAM) and trichostatin A (TSA) (Lv, Xu et al. 2013). There also exists a negative regulation by a deacetylase that regulates the deacetylation of the PKM2 K433 site. The deacetylase SIRT6 may deacetylate K433, suppressing PKM2s translocation to the nucleus and limiting its oncogenic functions, thus regulating cancer metabolism (Bhardwaj, Das, 2016). SIRT6 is a tumour suppressor, and the analysis of human cancer data shows the loss of this deacetylase in about 20% of all tumours (Guarente, 2014). Interestingly, SIRT6 also regulates the Warburg effect as it deacetylates histone H3 and acts as a co-repressor of c-Myc and HIF-1a, which represent other main drivers of the Warburg effect (Michishita et al., 2008, Sebastián et al., 2012, Zhong et al., 2010). It probably also regulates NF-κB dependent gene expression through the same mechanism (Kawahara et al., 2009). Finally, a study by van Meter et al. (2011) demonstrated that SIRT6 overexpression induces massive apoptosis in a variety of cancer cell lines but not in non-cancer cells, indicating SIRT6 as an attractive target for pharmacological activation in cancer treatment.

1.3.4 Interaction with the EGFR signalling pathway

A recent study has shown that PKM2 interacts with EGFR, which induces CSC properties in irradiation resistant cells. EGFR and PKM2 both translocate to the nucleus and may bind to each other. This interaction regulates the transcription of genes which promote the CSC phenotype, inducing invasion and metastasis (Shi et al., 2018). As mentioned above, EGFR was revealed as a potential phosphorylation target of PKM2-SAICAR. It has been confirmed that PKM2-SAICAR may phosphorylate the EGFR signalling downstream mediator ERK2, which leads to sustained ERK1/2 activation and cell proliferation (Keller et al., 2014). The activated ERK2 phosphorylates PKM2 in turn,

enhancing PKM2 protein kinase activity and nuclear translocation in a positive feedback-loop pattern, which then enhances the expression of glycolytic genes including PKM2. As such, nuclear PKM2 induces a positive feedback loop through an enhancement of its own gene expression (Yang et al., 2012c). The EGFR/ERK2-dependent promotion of PKM2s nuclear functions is illustrated in Figure 6.

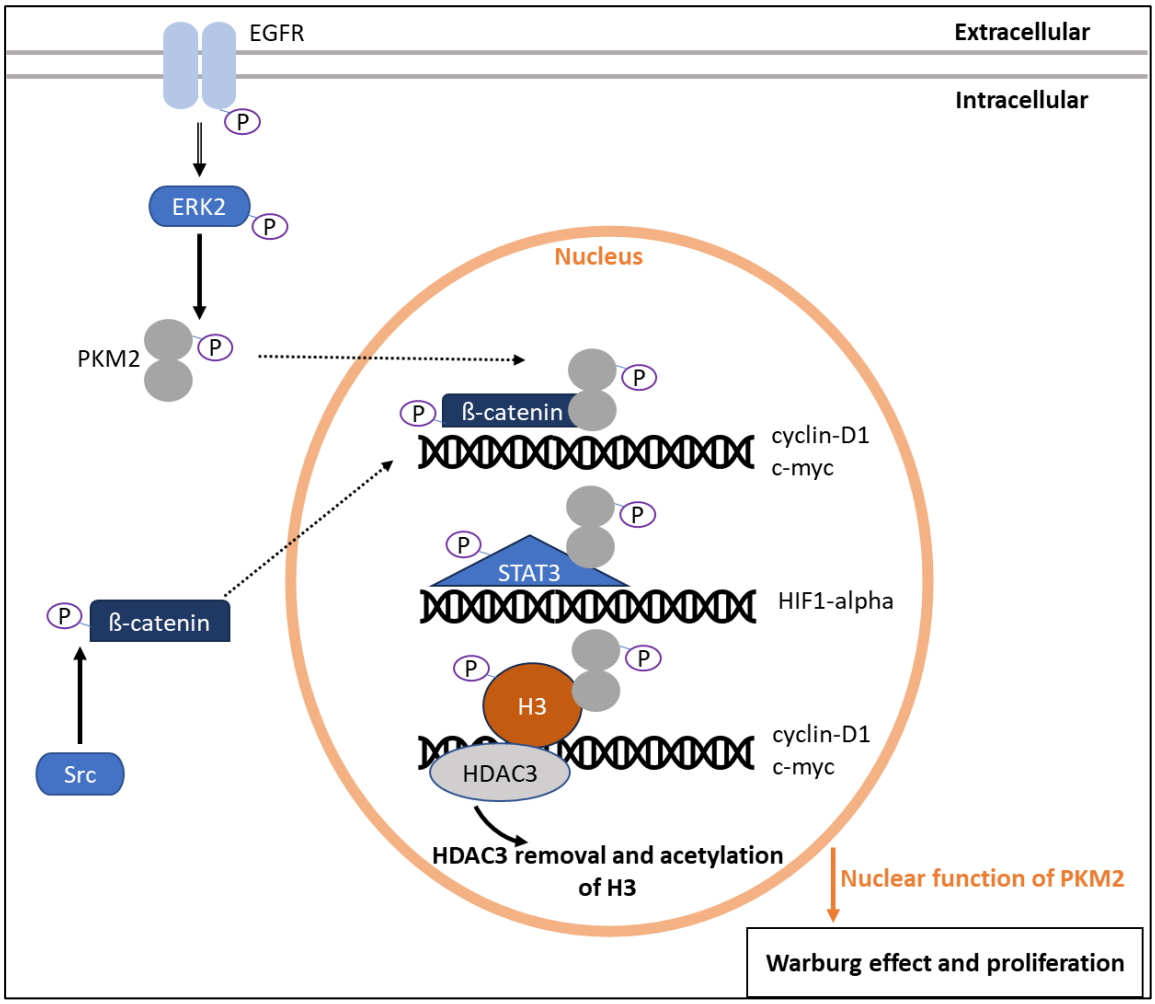


Figure 6. EGFR/ERK2-dependent promotion of PKM2s nuclear features (adapted from Wong et al., 2015). Activated ERK2 phosphorylates PKM2, which stimulates PKM2s nuclear translocation. In turn, nuclear PKM2 activates Src-phosphorylated ß-catenin and stimulates oncogenic and glycolytic gene expression (Wong et al., 2015).

EGFR/Akt signalling also transactivates β-catenin, which (Hu, Li, 2010) promotes the expression of genes important for cell proliferation or glycolytic metabolism, such as cyclin D1 or Myc (Yang et al., 2012a). As illustrated above, PKM2 activates Src-phosphorylated ß-catenin, which plays an important role in the establishment of both the glycolytic and the CSC phenotype of colon cancer cells (Vermeulen et al., 2010),

suggesting that PKM2 may enhance cancer stemness through this part of the EGFR signalling pathway (Li et al., 2014b).

Dimeric PKM2 also phosphorylates sites of Akt1 leading to Akt signalling activation promoting growth factor signalling (He et al., 2016). Moreover, the nuclear PKM2 directly phosphorylates Stat3, another downstream mediator of EGFR signalling with anti-apoptotic and proliferative traits. PKM2 might even be the reason for its constitutive activation in cancer cells (Gao et al., 2012, Klampfer, 2006). After EGF stimulation, the nuclear PKM2 also phosphorylates histone H3 and Bub3, which contribute to GBM tumour growth (Yang et al., 2012b). The PKM2-phosphorylated Bub3 has been shown to be required for EGFR-induced brain tumorigenesis in mice (Jiang et al., 2014a). Lastly, Hsu et al. (2016) showed that the extracellular PKM2 dimer might activate the EGFR signalling pathway in an autocrine manner, thus inducing cell proliferation.

Besides regulating the wild-type EGFR signalling, PKM2 may also regulate EGFR mutants in GBM. A study has showed that the suppression of nuclear PKM2 via the inhibition of SIRT6-regulated poly ADP ribose polymerase (PARP) induces sensitivity in EGFRvIII expressing glioblastoma and EGFR-T790M expressing lung cancers (Li et al., 2016, Mao et al., 2011). Moreover, Yang et al. (2016) showed that PKM2 may directly interact and stabilize the binding between mutant EGFRvIII and heat-shock protein 90 (HSP90), thus protecting mutant EGFRvIII from degradation.

In previous work in our laboratory, to examine whether PKM2 regulates EGFR signalling, PKM2 was overexpressed in U87 GBM and in A549 lung adenocarcinoma cells. Here, the activation of EGFR signalling and its downstream effectors were monitored in an experimental setup that included stimulation with EGF. The results showed that PKM2 enhances phosphorylation of EGFR and ERK1/2. Phosphorylated ERK1/2 (P-ERK1/2) is an indicator of the mitogen-activated protein kinase (MAPK) signalling which is associated with activated EGFR. However, since U87 is PTEN-deficient (Li et al., 1997), which leads to constitutive activation of the PI3K-pathway (Stambolic et al., 1998), differences in the phosphorylation of AKT could not be detected. These experiments, together with the aforementioned findings from the literature, provided the starting point for this work.

As briefly outlined in this Introduction, multiple regulatory pathways of PKM2 and PKM2-dependent regulatory mechanisms have been described in different model

systems. This emphasises the need for a better understanding of the GBM-specific and context-dependent function of this important regulator of cancer metabolism.

2 Aim of the study

Despite promising curative effect in the treatment of various cancers, the outcome of targeted therapy against the epidermal growth factor receptor (EGFR) in glioblastoma multiforme (GBM) is still disappointing. EGFR signalling has been shown receptive to regulation by various proteins, including the glycolytic enzyme and potential therapeutic target pyruvate kinase M2 (PKM2). PKM2 can interact both with EGFR and EGFR-binding proteins. Our pilot studies showed that PKM2 blocks the interaction between prolyl hydroxylase 3 (PHD3) and EGFR. Moreover, EGFR was revealed as a potential target of the PKM2 protein kinase.

The aim of this work was to elucidate whether and by what molecular mechanisms PKM2 may modify the activation of EGFR signalling and tumour growth. For this purpose, we generated stable PKM2-knockout glioblastoma cell lines with the CRISPR-Cas9-system and performed PKM2 overexpression rescue experiments *in vitro*. The experiments revealed that PKM2 overexpression enhances both EGFR phosphorylation and tumour cell growth upon EGF stimulation. To check whether the PKM2-induced tumour cell growth is mediated through EGFR signalling, we inhibited EGFR activity with its specific inhibitor erlotinib and analysed the tumour growth. In order to elucidate which post-translational modifications (PTMs) and features of PKM2 are essential for this regulation, a variety of PKM2 mutants were generated with site-directed mutagenesis. Finally, given the important role of acetylation in PKM2 activation, this study investigated whether the treatment with the deacetylase inhibitors trichostatin A (TSA) and nicotinamide (NAM) causes a PKM2-mediated upregulation of EGFR signalling.

3 Materials and Methods

3.1 Materials

3.1.1 Equipment

Product	Company
Biometra T3000 Thermocycler	Labrepco
Centrifuge JP Rotina 420	Hettich
Microcentrifuge Heraeus Fresco 17	Thermo Scientific
Microcentrifuge MiniStar silverline	vwr
Orbital Incubator Shaker	Eurotech Instruments & Calibration
Steri-Cult CO2-Incubator	Thermo Scientific
TCS SPE Microscope	Leica
NanoDrop 2000 Spectrophotometer	Thermo Scientific
AccuBlock™ Digital Dry Baths & Blocks	Labnet International
Casy Cell Counter and Analyzer TT	Biovendis
TriStar LB 941 Multimode Microplate	Berthold Technologies
Sonicater Sonipuls mini20	Bandelin
Synergy® Ultrapure Lab Water Systems	MilliporeSigma
Mini-Protean Tetra System	Bio-Rad Laboratories
Crurix 60 AGFA	Healthcare

3.1.2 Reagents

Reagents used for cell culturing	Company
Phosphate Buffered Saline (PBS)	Gibco
0,05% Trypsin-EDTA	Gibco
OptiMEM	Gibco
FuGene HD	Promega
Dimethyl Sulfoxide (DMSO)	Sigma
Amphotericin B	Sigma-Aldrich
Dulbecco's Modified Eagle Medium	Life Technologies
DMEM/F12	Life Technologies
Hepes	Life Technologies
B27 Supplement	Life Technologies
Fetal bovine serum (FBS)	Biochrom

Reagents used for cell culturing	Company
Gentamicin	Capricon
Puromycin	SigmaAldrich
FGF (# 100-18B)	Peprotech
EGF (# AF-100-15)	Peprotech

The growth factors EGF & FGF were reconstituted in 5 mM Tris Base and 0.1% BSA buffer for a concentration of 20 μg/mL with a pH of 7,6.

Inhibitory agents	Company
Erlotinib	Cayman
Trichostatin A (TSA)	Calbiochem
Nicotinamide (NAM)	Sigma

Reagents used for cell lysis	Company
p-APMSF Hydrochloride	Calbiochem
NP-40	Calbiochem
Protease inhibitor cocktail (#11697498001)	Roche
Sodium orthovanadate	Sigma
Sodium fluoride	Merck

Reagents used for Western Blotting	Company
ECL Western Blotting Substrate	Thermo Scientific
PageRuler Prestained Protein Ladder	Thermo Scientific
Spectra Multicolor High Range Protein Ladder	Thermo Scientific
PVDF-Membran 0,45μm 26,5cmX3,75m	Thermo Scientific
Ammonium persulfate (APS)	AppliChem
Potassium chloride	Sigma
Sodium phosphate	Sigma
0,1% Tween 20	Sigma
Methanol	Sigma

Reagents used for Western Blotting	Company
Isopropanol	Sigma
2-Mercaptoethanol	Sigma
Tetramethylethylendiamine (TEMED)	Roth
Milkpowder Blottin Grade	Roth
Bromphenol blue sodium salt	Roth
Tris	Roth
Glycin	Roth
SDS Ultra Pure	Roth
Acrylamid (30%)	Roth
Glycerol	Roth
Developer and Replenisher Unimatic-D	Calbe Chemie
Fixer and Replenisher Unimatic-F	Calbe Chemie
Potassium dihydrogen phosphate	Merck
DC Protein Assay Reagent A, B & S	Biorad

Reagents used for culturing bacteria	Company
Ampicillin	SigmaAldrich
SOC medium	Invitrogen
LB agar powder	Roth

Reagents used for site-directed mutagenesis	Company
Pfu Ultra HF DNA Polymerase	Stratagene
10 x Pfu Ultra HF Reaction Buffer	Agilent Technologies
Deoxynucleotide (dNTP) Solution Mix	New Englang Biolabs Inc.
Dpn1 restriction enzyme	Thermo Scientific

The used primers are listed under the chapter ´oligonucleotides´.

3.1.3 Buffers, mediums and solutions

All buffers and solutions containing water were prepared with deionized MilliQ water (dH2O) and stored at room temperature unless stated otherwise.

Solutions and buffers for Western Blotting:

4 x Sample buffer	Volume
SDS (10%)	40 mL
Tris-HCl (pH 6.8)	16 mL
Glycerol	20 mL
Deionized water	19 mL

The sample buffer was stored at -20°C.

10xSDS PAGE Running buffer	
Glycine	3,836 M
Tris Base	0,500 M
SDS (1%)	0,069 M
Deionized water	2 L

Transfer buffer	
Glycine	0,38 M
Tris Base	0,05 M
Methanol (15%)	300 mL
Deionized water	2 L

Washing buffer: 1l of 1xPBS and 10mL of Tween 20

Blocking buffer: 5% milk powder in washing buffer

Mediums for cell culture:

Tumour sphere medium (TSM)	**Volume**
DMEM/F12	500 mL
Hepes	2,5 mL
B27 Supplement	10 mL
Amphotericin B	5 mL
Gentamicin	500µL

Antibiotics	**Stock solution**	**Working concentration**
Ampicillin	100 mg/mL in dH2O	100 µg/mL
Puromycin	10 mg/mL in HEPES buffer	2 µg/mL

Mediums for cell lysis:

Laemmli lysis buffer	**For 100 mL**
Natriumfluorid	20 mM
EDTA	2 mM
SDS	9 mM
Tris-HCl (pH 7,5)	10 mM
Deionized water	Fill up to 100 mL

NP-40 buffer	
Tris Base	50 mM
Sodium chloride	150 mM
EDTA	4 mM
Glycerol	5%
NP40	1%

NP-40 buffer with protease and deacetylase inhibitors	**10 mL in total**
5x NP-40 buffer	2 mL
NP-40 (1%)	1 mL
Protease inhibitor cocktail	200 µL
p-APMSF (for a working concentration of 100 µM)	20 µL
TSA (for a working concentration of 10µM)	10 µL
NAM (for a working concentration of 20 µM)	50 µL
Sodium fluoride (for a working concentration of 10 mM)	200µL
Sodium orthovanadate (for a working concentration of 400 µM)	20µL
Deionized water	6,5 mL

The buffer was stored aliquoted at -20 °C.

Mediums used for bacterial selection in mini- & maxi-preps:

LB medium: 20 g of LB powder (Roth, Cat# X964.1) were dissolved in 1L dH2O and autoclaved at 120°C for 15 min. Autoclaved medium was stored at 4°C. Ampicillin was added right before the inoculation of bacteria for a final concentration of 100 µg/mL.
LB agar: 32 g of LB agar powder (Roth, Cat# X965.2) was dissolved in 1L dH2O, and autoclaved at 120°C for 15 min. After the solution cooled to approx. 40°C, Ampicillin was added for a final concentration of 100 µg/mL and the ready LB agar was poured into 10 cm Petri dishes. Ready agar plates were stored at 4°C.

3.1.4 Kits

Product	**Company**
PureLink Plasmid DNA Purification Maxiprep Kit	Invitrogen
PureLink Plasmid DNA Miniprep Kit	Invitrogen
Quick Change II Mutagenesis Kit	Agilent

3.1.5 Oligonucleotides

Oligonucleotides with the following **PKM2 point mutation**s and sequences (5´ → 3´) were all ordered from Sigma-Aldrich.

Y105F
Sense-Sequence CTGACCCCATCCTCTTCCGGCCCG
Antisense-Sequence CGGGCCGGAAGAGGATGGGGTCAG

K305Q
Sense GAGATTCCTGCAGAGCAGGTCTTCCTTGCTC
Antisense GAGCAAGGAAGACCTGCTCTGCAGGAATCTC

C358S
Sense CTGGATGGAGCCGACAGCATCATGCTGTCTG
Antisense CAGACAGCATGATGCTGTCGGCTCCATCCAG

K367M
Sense CTGGAGAAACAGCCATGGGGGACTATC
Antisense GATAGTCCCCCATGGCTGTTTCTCCAG

H391Y
Sense GGCTGCCATCTACTACTTGCAATTATTTGAGG
Antisense CCTCAAATAATTGCAAGTAGTAGATGGCAGCC

Q393K
Sense GCCATCTACCACTTGAAATTATTTGAGGAACTC

Antisense GAGTTCCTCAAATAATTTCAAGTGGTAGATGGC

P403A
Sense GCCGCCTGGCGGCCATTACCAGC
Antisense GCTGGTAATGGCCGCCAGGCGGC

P408A
Sense CATTACCAGCGACGCCACAGAAGCCAC
Antisense GTGGCTTCTGTGGCGTCGCTGGTAATG

K433E
Sense CATAATCGTCCTCACCGAGTCTGGCAGGTCTG
Antisense CAGACCTGCCAGACTCGGTGAGGACGATTATG

K433Q
Sense GGGGCCATAATCGTCCTCACCCAGTCTGGCAGGTCTGCTCACC
Antisense GGTGAGCAGACCTGCCAGACTGGGTGAGGACGATTATGGCCCC

K433R
Sense GGGGCCATAATCGTCCTCACCAGGTCTGGCAGGTCTGCTCACC
Antisense GGTGAGCAGACCTGCCAGACCTGGTGAGGACGATTATGGCCCC

3.1.6 Plasmids

Plasmid name	**Company**
hSpCas9-2A-Puro (PX459)	Addgene
pcDNA6.2/V5-DEST	Invitrogene

The plasmids PKM2-P403/408-V5, PKM2-K433E-V5, PKM2-K305Q-V5, PKM2-K367M-V5, PKM2-Y105F-V5, PKM2-K367M-V5, PKM2-K433Q-V5, PKM2-K433R-V5, PKM2-Q393K-V5, PKM2-C358S-V5 and PKM2-H391Y-V5 were created through site-directed mutagenesis using the oligonucleotides listed above.

The plasmid pcDNA-6.2-PKM2-WT-V5 was created by Dr. Huike Jiao. The pSpCas9(BB)-2A-Puro-SG1-PKM2 and pSpCas9(BB)-2A-Puro-SG6-PKM2 plasmids were created by Devendra Singh.

3.1.7 Antibodies

Primary antibody name	**Product number**	**Company**
EGFR (D38B1)	4267	Cell Signaling
pEGFR (Tyr1068)	3777	Cell Signaling

Primary antibody name	Product number	Company
PKM2	4053	Cell Signaling
V5	R960-25	Invitrogen
ERK2 (c-14)	sc-154	Santa Cruz
pERK1/2 (E-4)	sc-7383	Santa Cruz
anti-phosphotyrosine-HRP	HAM1676	R&D Systems
Tubulin	DLN09992	Dianova

The Ac-K433-PKM2 antibody has been generated by the research group that published the paper of Lv, Xu et al. 2013. The antibody has been obtained from the research group by Dr. Huike Jiao.

Secondary antibody name	Product number	Company
goat-anti-mouse-HRP	# 115-035-146	Jackson Immuno Research
goat-anti-rabbit-HRP	# 111-035-144	Jackson Immuno Research

3.1.8 Bacteria

One Shot™ Stbl3™ Chemically Competent *E. coli* from Invitrogen.

3.1.9 Cell lines

Cell lines	Company
A549 lung adenocarcinoma cell line human	Sigma
Uppsala 87 malignant glioma cell line human (U87 MG)	Sigma
U87-PX459 cell single clone	Self-created by CRISPR-knockout with the help of Dr. Huike Jiao (see chapter 3.2.2.6 Generation of stable knock-out cell lines)
U87-PKM2-Knockout-Depletion (KD) 1 cell line	
U87-PKM2-KD2 cell line	

3.1.10 Other materials

Product	Company
Pipetboy	Integra Biosciences
Pipets	Gilson, Middleton
Cell culture plates	Greiner Bio-One
Corning cell strainer	Sigma-Aldrich
CL-Xposure, 18 x 24cm	Thermo Scientific

3.2 Methods

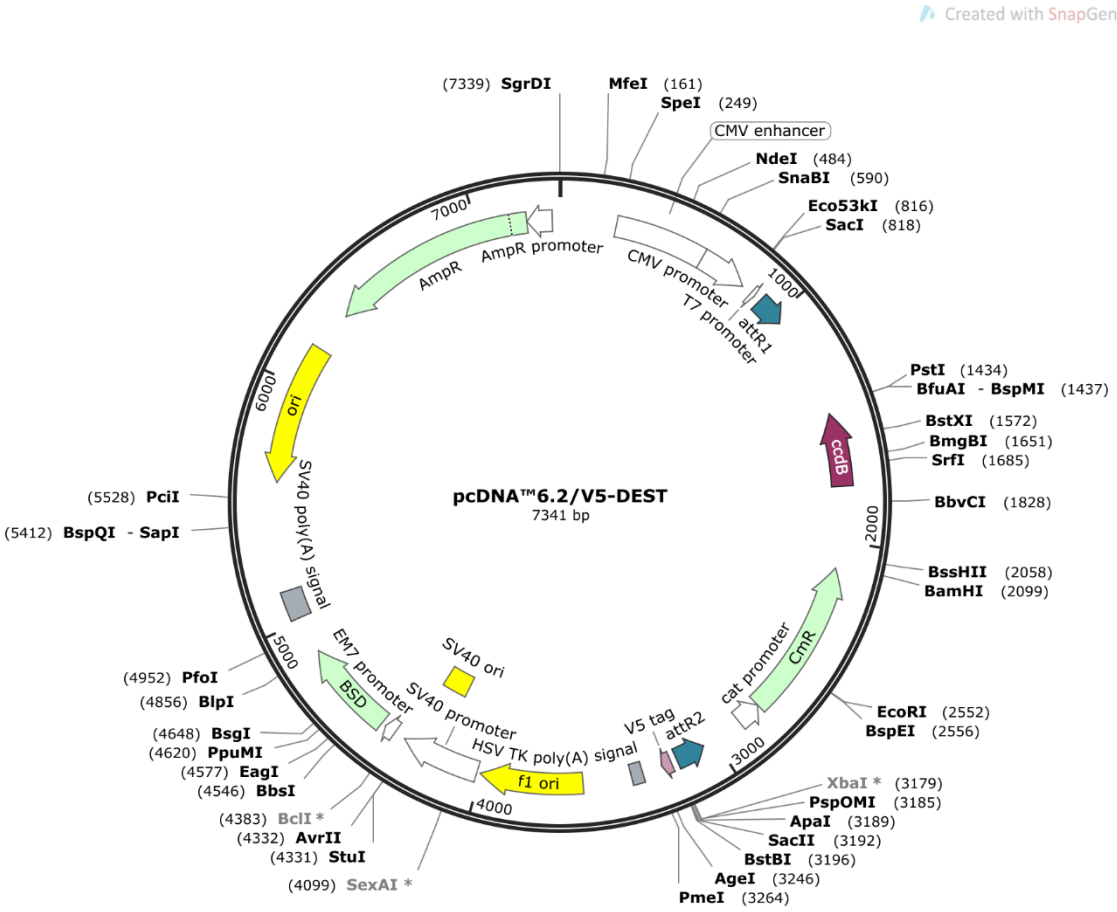
3.2.1 Preparation of plasmid DNA

3.2.1.1 Plasmid DNA constructs for PKM2 overexpression and knock-out

3.2.1.2 PCR-assisted site-directed mutagenesis

The coding region of the pyruvate kinase M2 (PKM2) was cloned into the commercially available plasmid pcDNA™6.2/V5-DEST with the Gateway™ LR Clonase™ II Enzyme mix following the manufacturer´s instructions by Dr. Huike Jiao. pcDNA™6.2/V5-DEST is a destination vector for high-level expression of C-terminally V5-tagged proteins in mammalian cells.

Plasmid map:



Sequencing primers:

T7 Promoter-Sequence 5´-TAATACGACTCACTATAGGG-3´
V5 Reverse-Sequence 5´-ACCGAGGAGAGGGTTAGGGAT-3´

Amplified PKM2 coding sequence (1596 bp):

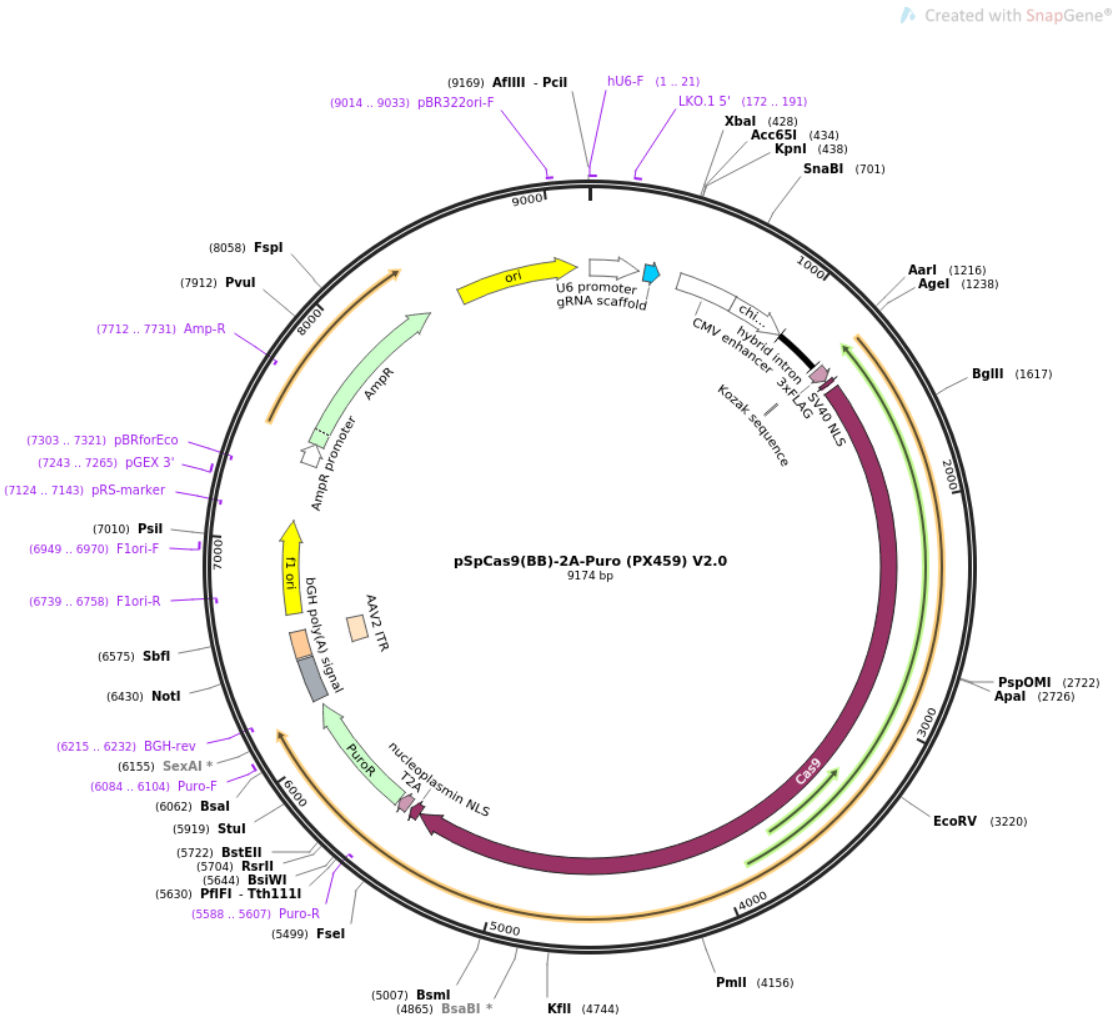
5´AAAAAAGTTGGCATGTCGAAGCCCCATAGTGAAGCCGGGACTGCCTTCATTCAGACCCAG
CAGCTGCACGCAGCCATGGCTGACACATTCCTGGAGCACATGTGCCGCCTGGACATTGATTC
ACCACCCATCACAGCCCGGAACACTGGCATCATCTGTACCATTGGCCCAGCTTCCCGATCAG
TGGAGACGTTGAAGGAGATGATTAAGTCTGGAATGAATGTGGCTCGTCTGAACTTCTCTCAT
GGAACTCATGAGTACCATGCGGAGACCATCAAGAATGTGCGCACAGCCACGGAAAGCTTTG
CTTCTGACCCCATCCTCTACCGGCCCGTTGCTGTGGCTCTAGACACTAAAGGACCTGAGATC
CGAACTGGGCTCATCAAGGGCAGCGGCACTGCAGAGGTGGAGCTGAAGAAGGGAGCCACTC
TCAAAATCACGCTGGATAACGCCTACATGGAAAAGTGTGACGAGAACATCCTGTGGCTGGA
CTACAAGAACATCTGCAAGGTGGTGGAAGTGGGCAGCAAGATCTACGTGGATGATGGGCTT
ATTTCTCTCCAGGTGAAGCAGAAAGGTGCCGACTTCCTGGTGACGGAGGTGGAAAATGGTG
GCTCCTTGGGCAGCAAGAAGGGTGTGAACCTTCCTGGGGCTGCTGTGGACTTGCCTGCTGTG
TCGGAGAAGGACATCCAGGATCTGAAGTTTGGGGTCGAGCAGGATGTTGATATGGTGTTTGC
GTCATTCATCCGCAAGGCATCTGATGTCCATGAAGTTAGGAAGGTCCTGGGAGAGAAGGGA

AAGAACATCAAGATTATCAGCAAAATCGAGAATCATGAGGGGGTTCGGAGGTTTGATGAAA
TCCTGGAGGCCAGTGATGGGATCATGGTGGCTCGTGGTGATCTAGGCATTGAGATTCCTGCA
GAGAAGGTCTTCCTTGCTCAGAAGATGATGATTGGACGGTGCAACCGAGCTGGGAAGCCTG
TCATCTGTGCTACTCAGATGCTGGAGAGCATGATCAAGAAGCCCCGCCCCACTCGGGCTGAA
GGCAGTGATGTGGCCAATGCAGTCCTGGATGGAGCCGACTGCATCATGCTGTCTGGAGAAA
CAGCCAAAGGGGACTATCCTCTGGAGGCTGTGCGCATGCAGCACCTGATTGCCCGTGAGGC
AGAGGCTGCCATCTACCACTTGCAATTATTTGAGGAACTCCGCCGCCTGGCGCCCATTACCA
GCGACCCCACAGAAGCCACCGCCGTGGGTGCCGTGGAGGCCTCCTTCAAGTGCTGCAGTGG
GGCCATAATCGTCCTCACCAAGTCTGGCAGGTCTGCTCACCAGGTGGCCAGATACCGCCCAC
GTGCCCCCATCATTGCTGTGACCCGGAATCCCCAGACAGCTCGTCAGGCCCACCTGTACCGT
GGCATCTTCCCTGTGCTGTGCAAGGACCCAGTCCAGGAGGCCTGGGCTGAGGACGTGGACCT
CCGGGTGAACTTTGCCATGAATGTTGGCAAGGCCCGAGGCTTCTTCAAGAAGGGAGATGTG
GTCATTGTGCTGACCGGATGGCGCCCTGGCTCCGGCTTCACCAACACCATGCGTGTTGTTCCT
GTGCCGTGCCCAACTTTCATGTACAAAGTGGTG 3´

The stop codon was removed in order to add the fusion V5 tag in frame with the c-terminal domain of the protein.

The following CRISPR PKM2-Knockout constructs were created by Devendra Singh:

Plasmid map of the control vector pSpCas9(BB)-2A-Puro (PX459)



Coding sequence of sg1-PKM2 (used for the creation of the PKM2-KD1 U87 cells):

5´TTGGAATTAATTTGACTGTAAACACAAAGATATTAGTACAAAATACGTGACGTAGAAAGT
AATAATTTCTTGGGTAGTTTGCAGTTTTAAAATTATGTTTTAAAATGGACTATCATATGCTTA
CCGTAACTTGAAAGTATTTCGATTTCTTGGCTTTATATATCTTGTGGAAAGGACGAAACACC
GATTTGAGGAACTCCGCCGCCGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCC
GTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTTTTTTGTTTTAGAGCTAGAAATAGCA
AGTTAAAATAAGGCTAGTCCGTTTTTAGCGCGTGCGCCAATTCTGCAGACAAATGGCTCTAG
AGGTACCCGTTACATAACTTACGGTAAATGGCCCGCCTGGCTGACCGCCCAACGACCCCCGC
CCATTGACGTCAATAGTAACGCCAATAGGGACTTTCCATTGACGTCAATGGGTGGAGTATTT
ACGGTAAACTGCCCACTTGGCAGTACATCAAGTGTATCATATGCCAAGTACGCCCCCTATTG
ACGTCAATGACGGTAAATGGCCCGCCTGGCATTGTGCCCAGTACATGACCTTATGGGACTTT
CCTACTTGGCAGTACATCTACGTATTAGTCATCGCTATTACCATGGTCGAGGTGAGCCCCAC
GTTCTGCTTCACTCTCCCCATCTCCCCCCCCTCCCCACCCCCAATTTTGTATTTATTTATTTTT
3´

Sequence of forward sgRNA-Insert

Vector backbone

Coding sequence of sg6-PKM2 (used for the creation of the PKM2-KD2 U87 cells):

5´AGAGAGATAATTGGAATTAATTTGACTGTAAACACAAAGATATTAGTACAAAATACGTGA
CGTAGAAAGTAATAATTTCTTGGGTAGTTTGCAGTTTTAAAATTATGTTTTAAAATGGACTAT
CATATGCTTACCGTAACTTGAAAGTATTTCGATTTCTTGGCTTTATATATCTTGTGGAAAGGA
CGAAACACCGGTCGCTGGTAATGGGCGCCGTTTTAGAGCTAGAAATAGCAAGTTAAAATAA
GGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTTTTTTGTTTTAGAGCTA
GAAATAGCAAGTTAAAATAAGGCTAGTCCGTTTTTAGCGCGTGCGCCAATTCTGCAGACAAA
TGGCTCTAGAGGTACCCGTTACATAACTTACGGTAAATGGCCCGCCTGGCTGACCGCCCAAC
GACCCCCGCCCATTGACGTCAATAGTAACGCCAATAGGGACTTTCCATTGACGTCAATGGGT
GGAGTATTTACGGTAAACTGCCCACTTGGCAGTACATCAAGTGTATCATATGCCAAGTACGC
CCCCTATTGACGTCAATGACGGTAAATGGCCCGCCTGGCATTGTGCCCAGTACATGACCTTA
TGGGACTTTCCTACTTGGCAGTACATCTACGTATTAGTCATCGCTATTACCATGGTCGAGGTG
AGCCCCACGTTCTGCTTCACTCTCCCCATCTCCCCCCCCTCCCCACCCCCAATTTTGTATTTAT
TTATTTTTT 3´

Sequence of forward sgRNA-Insert
Vector backbone

In order to examine whether change of specific amino acids and associated posttranslational modifications in PKM2 has an effect on EGFR signalling, we created specific plasmids with site-directed mutagenesis. The oligonucleotides with the desired point mutations were ordered at Microsynth Seqlab. The point mutations were constructed with the Quick Change II Mutagenesis Kit (Agilent) following to the manufacturer´s instructions by inserting oligonucleotides with the specific point mutations, in the original C-terminally V5-tagged (wt) PKM2 construct.

The PCR was performed with the Biometra T3000 Thermal Cycler (analytikjena) using the following program:

Initial denaturation 95˚C, 30 sec

Denaturation 95˚C, 30 sec

Primer annealing 55˚C, 1 min

18 cycles Elongation 68°C, 1 min/kb of plasmid length

The amplified plasmid DNA was transformed into One Shot™ Stbl3™ Chemically Competent *E. coli* bacteria (Invitrogen) (see 3.2.1.3). The bacteria were plated on LB agar

containing the antibiotic ampicillin with a concentration of 100 µg/mL. Plasmid DNA was purified (see 3.2.1.4) and the presence of the desired point mutation was confirmed with DNA sequencing (see 3.2.1.6).

The point mutations inserted in the PKM2 coding region of the pcDNA6.2-PKM2-V5 plasmid were Q393K, K433Q, K433R, K433E, K305Q, C358S, H391Y, P403/408A, K367M and Y105F (for the oligonucleotides employed in the mutagenesis see chapter 3.1.5).

3.2.1.3 Transformation of competent bacteria

The procedure of transformation plants the desired plasmid DNA into the bacteria, where the amount of it increases as the bacteria containing the ampicillin antibiotic resistance breed. For the transformation One Shot™ Stbl3™ Chemically Competent *E. coli* bacteria (Invitrogen) were slowly thawed on ice. 50 µL of the plasmid were given to 100 µL of competent cells. The mixture incubated 30 min on ice. The bacteria incorporated the plasmid during a heat shock at 42˚C for 45 seconds. Subsequently, the cells were put for 2 min on ice. 250 µL of SOC-medium was added into the mixture. Then the mixture was shaken for 40 min at 225 rpm with Orbital Incubator Shaker. Subsequently, the bacteria were streaked on prewarmed LB-agar plates that contained the antibiotic Ampicillin with a concentration of 100 µg/mL and breeded for 12-16 hours at 37 °C. For the first 30 min of the breeding we left the dishes upside-down in order to dry them up. Single colonies were picked and put into 5 mL of liquid LB-medium that contained Ampicillin in a concentration of 100 µg/mL and breeded for up to 16 hours at 37°C shaked at 225 rpm with Orbital Incubator Shaker.

3.2.1.4 Small-scale plasmid DNA isolation

The isolation of the plasmid DNA from the transformed *E. coli* bacteria was performed with a PureLink Quick Plasmid DNA Miniprep Kit (Invitrogen) according to the manufacturer's instruction. For this procedure 5 mL of overnight bacterial culture in liquid LB-medium that contained Ampicillin in a concentration of 100 µg/mL was used.

Contrary to the instructions, centrifugation of the plasmid was performed at 4700 rpm instead of 12000 rpm and two times as long as recommended with Microcentrifuge Heraeus Fresco 17. The concentration of the purified plasmid DNA was quantified in a spectrophotometer by measuring absorbance at a wavelength of 260 nm. The ratio of the

absorbance at 260 and 280 nm (OD260/280) was used to evaluate the purity of the plasmids. An OD260/280 ratio between 1.8 and 2.0 was presumed to be a high-quality sample. The purified plasmid DNA was dissolved in nuclease free water and stored in a –20°C freezer.

3.2.1.5 Large-scale plasmid DNA isolation

In order to get sufficient amounts of plasmid DNA for the experiments that involve transient transfections, we used 200 mL of adequately transformed overnight bacterial culture in liquid LB-medium that contained Ampicillin in a concentration of 100 µg/mL. The isolation of the plasmid DNA from the E. coli was performed with a Purelink HiPure Plasmid DNA Purification Kit (Invitrogen) according to the manufacturer´s instruction. Contrary to the instructions, centrifugation of the plasmid was performed at 4700 rpm instead of 12000 rpm and two times as long as recommended with Microcentrifuge Heraeus Fresco 17. The concentration of the in nuclease free water dissolved purified plasmid DNA was quantified in a spectrophotometer by measuring absorbance at a wavelength of 260 nm. The ratio of the absorbance at 260 and 280 nm (OD260/280) was used to evaluate the purity of the plasmids. An OD260/280 ratio between 1.8 and 2.0 was presumed to be a high-quality sample. The plasmid DNA was then diluted with nuclease free water to a concentration between 1 µg/µL and 4 µg/µL, and stored in a –20°C freezer.

3.2.1.6 Plasmid DNA sequencing

The selection´s accuracy of the PKM2 plasmids containing the desired point mutation was confirmed by DNA sequencing. For this purpose, nuclease free water with 2 µg of plasmid DNA that we previously purified with the small scale plasmidic isolation method (see 3.2.1.4) was sent for sequencing to Microsynth Seqlab Company (Göttingen, Germany). The sequences that we received from Seqlab were aligned with a reference sequence (Genebank Database) using the BLAST (Basic Local Alignment Search Tool) bioinformatic program.

3.2.1.7 Bacteria storage

To store bacterial clones, 500 µL bacterial culture was mixed with 500 µL of 80% glycerol and frozen at -80°C.

3.2.2 Cell culture

3.2.2.1 Propagation of cells

Cells were cultured in 10 mL DMEM/10% FBS medium at 37°C, 5% CO2 and normoxia in 10cm tissue culture dishes, unless specified otherwise. Cells were grown no longer than one month after thawing and regularly passaged. The medium was changed every 2-3 days.

3.2.2.2 Passaging of cells

In general, cells were cultured until 80% of confluency was reached. For passaging the cells were washed with 1xPBS and digested with 3 mL 0.05% trypsin/EDTA solution for 3 min. After the cells detached from the dish, the trypsin was inactivated by the addition of 4 mL DMEM/10%FBS medium. Then the cell suspension was centrifuged at 1000 rpm with Centrifuge JP Rotina 420. The supernatant was discarded, cells were resuspended in fresh medium and between 0,8 million and 2 million cells were seeded to a single dish. For all experiments 4 million cells were seeded at the beginning, unless specified otherwise.

3.2.2.3 Counting and seeding of cells

100 µL of cell suspension were diluted in 10 mL of Casy ton buffer and analyzed with the Casy cell counter following the manufacturer´s instructions. As dead cells possess ruptured cell membranes this method allows an assessment of the cells vital state. For suspension condition 2 million cells per dish were seeded.

3.2.2.4 Freezing of cells

The cells were resuspended in cryomedium (DMEM with 20% DMSO and DMEM/10%FBS medium 500 µL each), and aliquoted in 1 mL cryovials. The vials were gradually cooled in cryoboxes at -80°C and for long term storage they were afterwards transferred to liquid nitrogen.

3.2.2.5 Thawing of frozen cells

The cells in the cryovials were thawed in a water bath at 37 C. Immediately after thawing they were put to 10 cm wide tissue culture dishes filled with 10 mL of DMEM/10%FBS medium. The medium was changed no later than 24h after thawing.

3.2.2.6 Generation of stable knock-out cell lines

U87 cells were transfected with the control vector pSpCas9(BB)-2A-Puro (PX459) and the two different CRISPR knock-out constructs pSpCas9(BB)-2A-Puro(PX459)-sg1-PKM2 and pSpCas9(BB)-2A-Puro(PX459)-sg6-PKM2 by Dr. Huike Jiao. One dish of the non-transfected cells was kept as a control of efficiency of the puromycin selection. 24h after the transfection, the puromycin selection has been started. The selection lasted 4 days. After the puromycin selection the clones were propagated until there were enough cells to freeze and to test for protein expression. The success of the knock-out has been assessed through cell lysing and Western blotting.

3.2.2.7 Transient transfection

The cells were always transfected 24h after passaging. For one transfection, an amount of 16 ug plasmid, 600 µL OPTI-MEM medium and 48 uL FuGene reagent were used and incubated 5 min at room temperature. Thereafter, the mixture was given drop by drop to the cells.

The concept for the experiments that included transient transfection was the following: Passaging and seeding of the cells in adherent condition on the first day. Transfecting the cells with the plasmid on the second day. Seeding the cells in suspension condition on the third day. Stimulating the cells with EGF after a starvation period of 24h on the fourth day.

3.2.2.8 Cell starvation and stimulation with EGF

After splitting to suspended condition, the cells were starved in TSM medium for 24h, and stimulated with EGF (20 ng/mL) right before the protein isolation process.

3.2.2.9 Protein isolation from cells

The protein isolation from cells for the experiments involving deacetylase inhibitors occurred with the NP40 buffer with protease and deacetylase inhibitors. For all other experiments Laemmli buffer was used.

The cells in the medium were centrifuged at 2500 rpm at 4°C for 1 min in 50 mL falcons each filled with 10mL ice-cold PBS with Centrifuge JP Rotina 420. Afterwards the supernatant was dumped, and the cell pellets were resuspended with 75 µl of a lysis buffer on ice into 1,5 mL eppendorf tubes. After harvesting the cells in Laemmli buffer (but not in the NP40 buffer) cells were heat shocked at 95 degrees for 5 min. Finally, the lysates were vortexed and sonicated at 90% intensity (0.5 sec / 0.5 sec sonication-rest intervals) for 20 sec with Sonopuls (Bandelin). The samples were stored at -20 °C.

3.2.3 Protein quantification

Protein concentration was double determined by Biorad DC Protein Assay Kit according to manufacturer´s instructions by a microplate reader. The lysis buffer was used as control. The assay is based on the Lowry method (Lowry et al., 1951), and the absorbance was measured at the light wavelength of 750 nm. The concentration of the protein samples was determined in duplicates (5 µL each) by comparison with a standard curve. The standard curve was generated based on the absorbance values of different dilutions of BSA.

3.2.4 SDS polyacrylamide gel electrophoresis

With sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) one can separate proteins basing on their molecular weight in a gel matrix. For the SDS-PAGE gels composed of two gel phases were prepared. In the upper stacking gel the concentration of the proteins gets increased. The anionic SDS-coated proteins move there between chloride and glycine ions, thus increasing their concentration. In the lower part of the gel the proteins get separated depending on their molecular weight. The smaller proteins move faster towards the cathode, whereas the larger proteins move slower as they get more stuck between the pores of the gel. For the proteins of interest one has to choose a gel with a corresponding pore size. Accordingly, gels containing an acrylamide proportion of 8% were created with the following recipe.

Separating gel	Volume
Lower buffer (pH 8,8)	2,6 mL
Acrylamid (30%)	2,7 mL
APS (10%)	50µL
TEMED	5µL
dH2O	4,65 mL

Stacking gel	Volume
Upper buffer (pH 6,8)	1,3 mL
Acrylamid (30%)	0,65 mL
APS (10%)	50µL
TEMED	5µL
dH2O	3,05 mL

While the separating gel has polymerized in the glass plates after around 30 min, it was covered with isopropanol. The isopropanol was then washed out with deionized water, and the stacking gel filled in together with a comb.

The protein samples were diluted with sample buffer at a volume ratio of 3:1. The sample buffer consisted of 800 µL 4 x SDS sample buffer, 200 µL 1% bromphenol blue and 50 uL ß-mercaptoethanol. β-mercaptoethanol was used for disrupting disulfide bridges between the proteins and bromphenol blue was used for staining the samples.

The samples were shortly vortexed before loading each 30 µg of proteins into the gel pockets filled with the Running buffer. Electrophoresis was carried out first at 80V until the band of the Prestained Protein Ladder got into the separating gel, and then increased to 130V till the proteins got adequately separated in the gel.

3.2.5 Western immunoblotting

The proteins on the separating gels were transferred on a polyvinylidene fluoride (PVDF) membrane in a blotting apparatus. For this purpose, PVDF membranes were put into 100% methanol, and two whatman papers into transfer buffer. The PVDF membranes were then transferred into the transfer buffer, too. Afterwards, inside the transfer buffer the separating gel was put on top of the two whatman papers, one PVDF membrane was put on top of the gel, and one whatman paper was put on top of the PVDF membrane. Before putting this “sandwich” to the blotting apparatus, air bubbles were removed by applying consistant pressure from one side to the other inside the transfer buffer. The transfer of the proteins from the gel to the PVDF membrane was then conducted by applying an electric current of 250mA to the blotting apparatus for 2 hours.

All of the following incubations of the membrane were performed under constant smooth shaking of the membrane. After the transfer the membrane was put into a solution

consisting to 5% of milk powder and to 95% of washing buffer for 1 hour. Each PVDF-membrane was cut and incubated in a solution containing either the primary antibodies against the phosphorylated antibodies and tubulin, or the antibodies against EGFR, ERK-2 and V5/PKM2 fitting to the expected molecular weight of the protein on the membrane at 4°C over night. The next day, the membrane was washed three times for 8 min each with wash buffer, and then incubated in the solution with a corresponding horseradish peroxidase (HRP-)-coupled secondary antibody for 1,5 hours at room temperature. After washing the membrane again three times for 8 min each, the membrane was put on an unruffled foil and the membrane was soaked in a few drops of ECL solution for 3 min under light protection. The signals on the membrane were recorded with Crurix 60 AGFA.

3.2.6 Sphere formation assay

The sphere formation assay (SFA) is a functional *in vitro* assay in which a 3-D tumour culture matrix is maintained under specific serum-independent conditions. It replicates the growth characteristics of tumours *in vivo*. The seeded cancer cells that have self-renewing properties form 3D tumour spheroids, which resemble the tumour microenvironment *in vivo*. Therefore, the SFA is being used for estimation of the stem cell population in cancer cell lines, and also for drug screenings of drugs that can specifically target cancer stem-like cells.

For the sphere formation assay the cells were cultured until reaching around 80% of confluency. The cells were washed with 1xPBS and digested with 3 mL 0.05% trypsin/EDTA solution for 3 min. After the cells detached from the dish, the trypsin was inactivated by the addition of 4 mL DMEM/10%FBS medium. Then the cell suspension was centrifuged at 1000 rpm with Centrifuge JP Rotina 420. After the supernatant was discarded, the cells were resuspended in TSM medium and put through a 70µm mesh. After cell counting, 2 mL of TSM medium containing 500 cells per µL were given to each well of a 6-well suspension cell culture plate. The growth factors EGF & FBF (20 ng/mL) were given to each well, and then the cells were left for 5 days to grow. Finally, the spheres were counted using the Leica TCS SPE microscope with the 40x immersion objective, assuming at least six connected cells as one sphere. The sphere formation units (SFU) were calculated based on the article of Reynolds & Rietze, 2005.

3.2.7 Statistics

Data analysis was performed by using Excel 2016 (Microsoft). Each experiment was performed at least three times on independent occasions. The results were expressed as means ± standard deviation. Experimental groups were compared with controls using the Student´s t-test. The statistical significance was assumed at the following p-values: $p<0.05$ (*), $p<0.01$ (**), and $p<0.001$ (***).

4 Results

4.1 Knockout of PKM2 suppresses EGFR signalling

In order to assess the effect of PKM2 on EGFR signalling in U87 cells, which have high cellular PKM2 levels, we established PKM2 knockout-depletion U87 cells PKM2-sg1 and PKM2-sg6 (U87-PKM2-KD1 and U87-PKM2-KD2; see chapter 3.2.2.6). Generation of PKM2 knockout in U87 by using CRISPR-Cas9 was performed by Dr. Huike Jiao. Both sgRNAs targeting PKM2 showed abrogated activation of EGFR, determined by phosphorylated EGFR and ERK1/2 (Figure 7). The suppression of EGFR-signalling was also seen subsequent to transient knockdown by PKM2-specific siRNA, confirming the results of the KO experiments. The experiments with siRNA have been performed by Dr. Huike Jiao (data not shown).

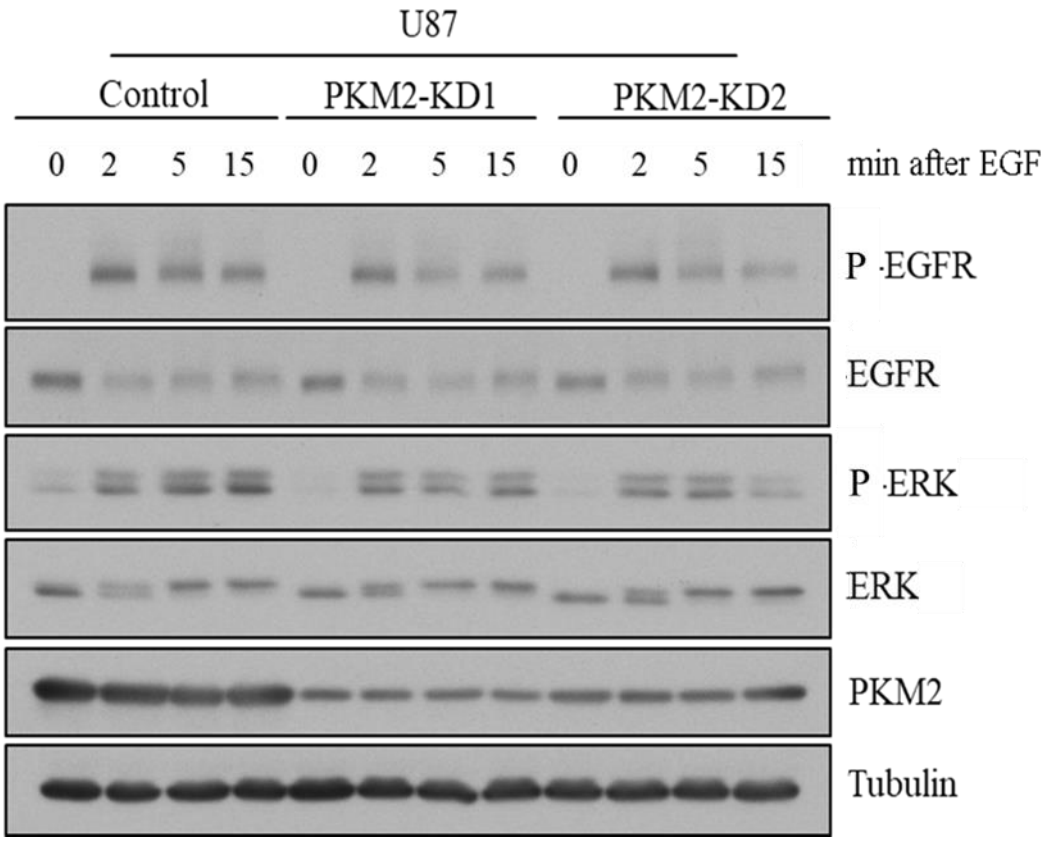


Figure 7. Knockout of PKM2 suppresses EGFR-signalling. Immunoblot of (P-)EGFR, (P-)ERK and PKM2 of PKM2-knockout U87 cells that were non-stimulated or stimulated with EGF (20 ng/mL) for 2, 5 or 15 min. Tubulin served as internal loading control.

4.2 PKM2 increases EGFR signalling

Next, we overexpressed PKM2 in the PKM2 knockout cells and evaluated the effect of PKM2 on EGFR signalling. The results show a substantial enhancement of EGFR signalling induced by the overexpression of PKM2 (Figure 8).

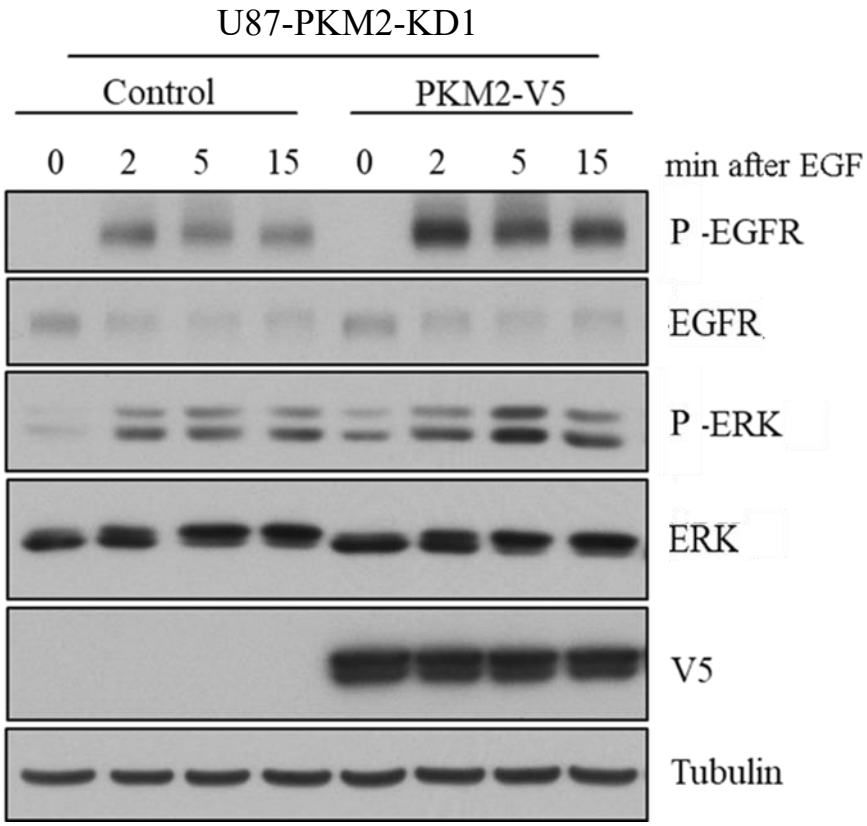


Figure 8. PKM2 increases EGFR signalling. Immunoblots of (P-)EGFR, (P-)ERK and V5-tagged PKM2 in PKM2 knockout U87 cells with expression of empty control or V5-tagged PKM2 which were non-stimulated or stimulated with EGF (20 ng/mL) for 2 min, 5 min and or 15 minutes two days after PKM2 transient transfection. Tubulin served as internal loading control. P-EGFR and P-ERK-1/2 protein levels positively correlate with PKM2 levels.

4.3 PKM2 affects tumour cell growth

4.3.1 Knockout of PKM2 suppresses tumour cell growth

The effect of PKM2 on tumour growth was assessed with a sphere forming assay (SFA). As the number of tumour spheres is a parameter of clonal growth, the SFA is a parameter to assess tumour cell growth *in vivo* (see chapter 3.2.6). As shown in Figure 9.1, stable knockout of PKM2 significantly decreased the number of spheres.

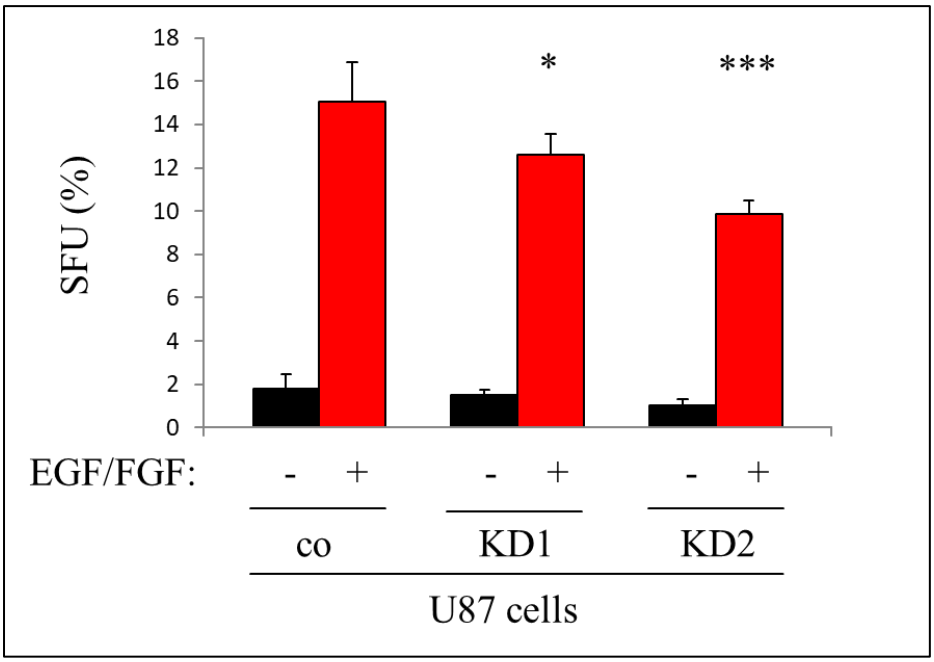


Figure 9.1. Depletion of PKM2 inhibits tumour cell growth. U87 with CRISPR-Cas9 knockout of PKM2 by 2 different sgRNA were cultured as spheroids in B27-supplemented serum-free medium+EGF/FGF (20 ng/mL) for five days. The number of spheroids was quantified (n=6).

4.3.2 Overexpression of PKM2 promotes tumour cell growth

We then overexpressed PKM2 in the PKM2 knockout cells and evaluated the effect of PKM2 on tumour cell growth. The results show that PKM2 overexpression prominently increased tumour sphere formation (Figure 9.2).

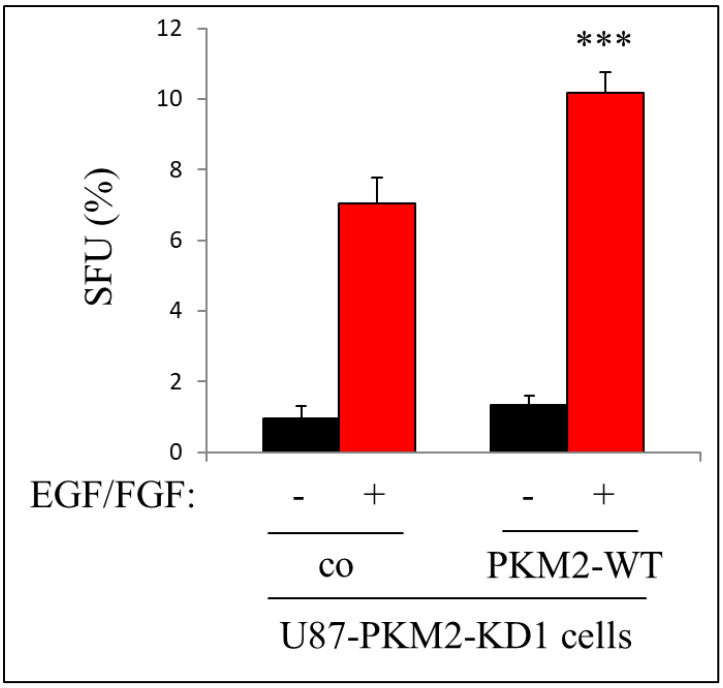


Figure 9.2. Overexpression of PKM2 promotes tumour cell growth. U87 with CRISPR-Cas9 knockout of PKM2 were transiently transfected with empty-vector or PKM2-V5 and cultured as spheroids in B27-supplemented serum-free medium+EGF/FGF (20 ng/mL) for five days. The number of spheroids was quantified (n=6).

4.4 EGFR mediates the growth-promoting effect of PKM2

Next, we investigated whether the growth-promoting effects of PKM2 are functionally dependent on increased EGFR signalling. Our results showed that inhibition of EGFR signalling by erlotinib can abrogate PKM2-overexpression promoted tumour cell growth (Figure 10). These results suggest that PKM2 promotes tumour cell growth through EGFR activation.

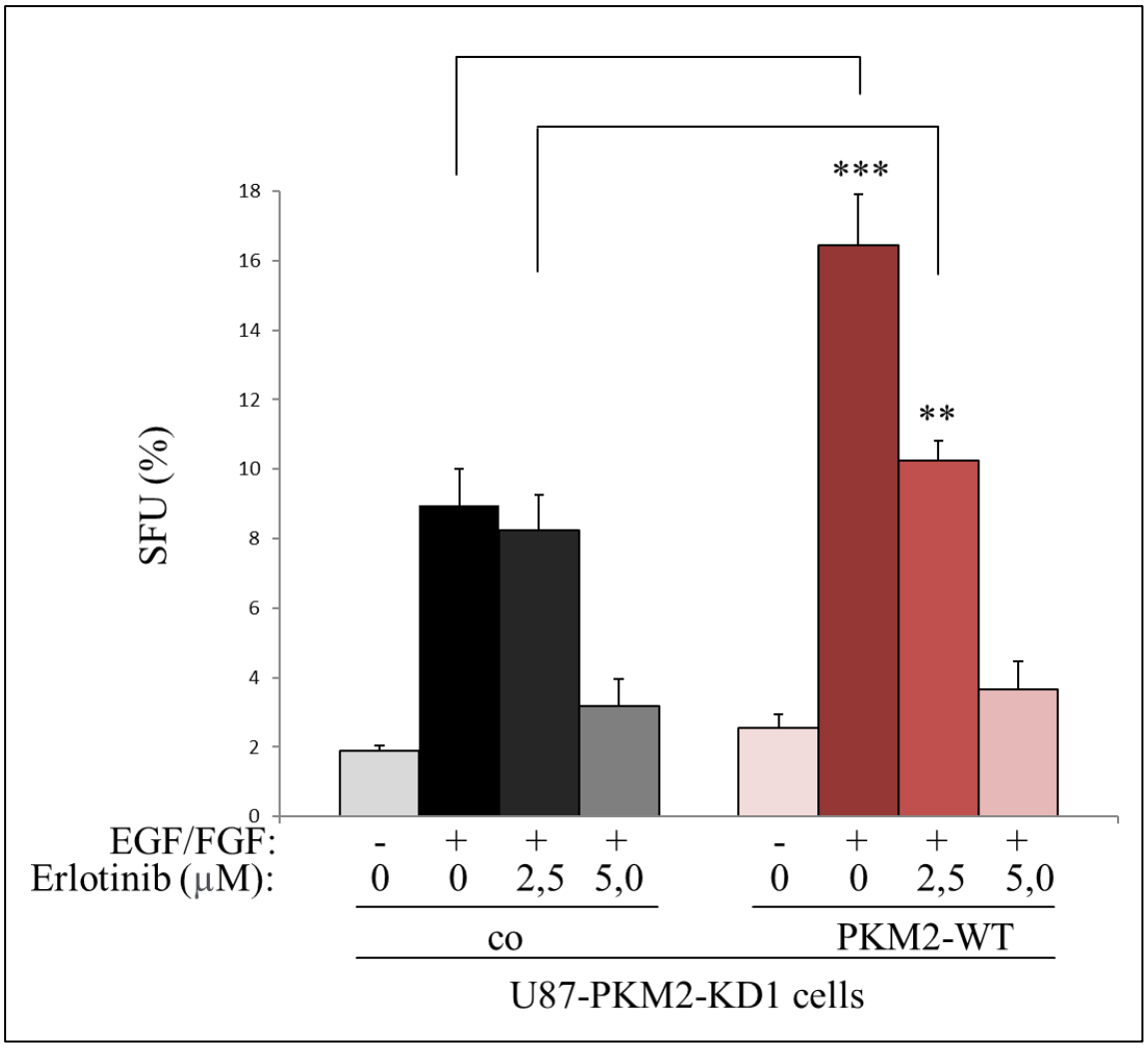


Figure 10. U87-CRISPR-construct cells with stable PKM2-knockout were cultured as spheroids in B27-supplemented serum-free medium+EGF/FGF (20 ng/mL) one day after transient overexpression of PKM2-WT. Where indicated, cells were pretreated with the EGFR inhibitor Erlotinib (2,5 µM and 5 µM). The number of spheroids was quantified (n=6).

4.5 Pyruvate kinase dead mutant of PKM2 (K367M) increases EGFR-signalling

Next, PKM2-mutants K367M, Q393K, K433R, K433Q, K433E, K305Q, C358S, H391Y, P403/408A and Y105F were created by site-directed mutagenesis (see chapter 3.2.1.2). The enhancement of EGFR signalling was also induced by the overexpression of the pyruvate kinase dead PKM2-mutant K367M (Figure 11). Initial experiments with the PKM2 mutants K433E, Y105F, K305Q or C358S showed similar results, but require further investigations to draw conclusions and they are therefore not displayed here.

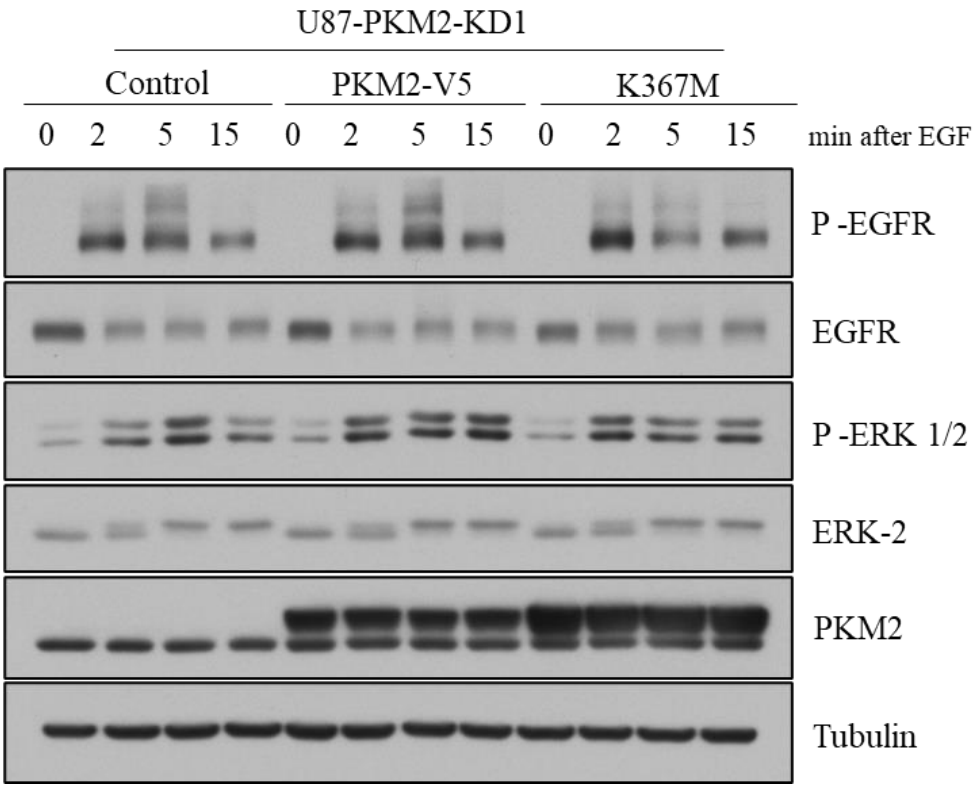


Figure 11. Immunoblot for (P-)EGFR, (P-)ERK and PKM2 of U87-CRISPR-construct cells with stable PKM2-knockout that were non-stimulated or stimulated with EGF (20 ng/ml) for 2, 5 and 15 min two days after transient overexpression of PKM2-WT and pyruvate kinase dead mutant of PKM2 K367M. Tubulin served as internal loading control.

4.6 Protein kinase dead mutants of PKM2 (Q393K, K433R) do not enhance EGFR-signalling

4.6.1 PKM2-Q393K mutant does not enhance EGFR signalling

The PKM2-mutants PKM2-Q393K, PKM2-P403/408A and PKM2-K433R affected the EGFR signalling differently compared to PKM2-wildtype. First, to examine whether the protein kinase dead PKM2 mutant Q393K can enhance EGFR signalling, we overexpressed it in parental U87 GBM cells, and checked whether the phosphorylation of EGFR was augmented.

In contrast to wild-type PKM2, in U87 cells transiently transfected with PKM2 mutant Q393K, a SAICAR-insensitive mutant with reduced pyruvate and protein kinase activities, both the phosphorylated EGFR and phosphorylated ERK1/2 levels were not comparably enhanced upon stimulation with EGF, suggesting that the Q393 site of PKM2

is necessary for the enhancement of EGFR signalling (Figure 12.1). Hence, it might be the protein kinase activity of PKM2 that is necessary for PKM2-mediated increased EGFR phosphorylation. Experiments with PKM2-Q393K in U87-PKM2-KD cells were performed by Dr. Huike Jiao and confirmed the result (Figure 12.2). Additional experiments with overexpression of hydroxylation site mutant PKM2-P403/408A in U87-PKM2-KD cells were also performed by Dr. Huike Jiao and led to similar results (data not shown).

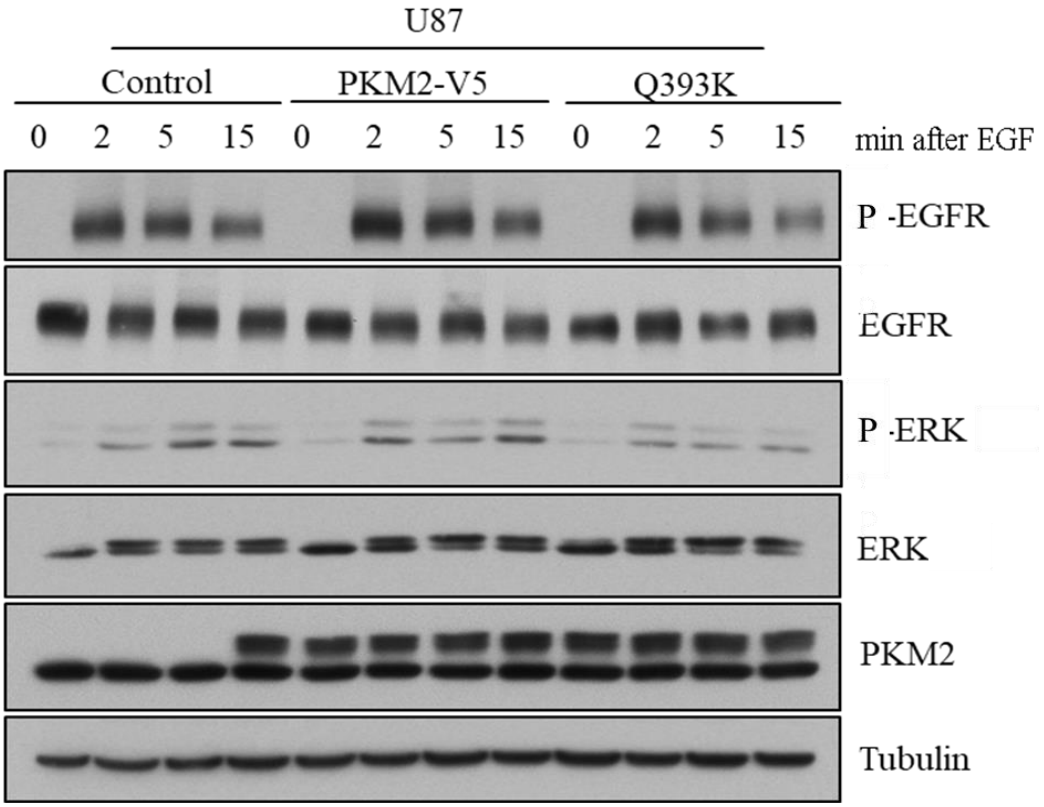


Figure 12.1. Immunoblot for (P-)EGFR, (P-)ERK and PKM2 of U87 cells that were non-stimulated or stimulated with EGF (20 ng/ml) for 2, 5 and 15 min two days after transient overexpression of PKM2-WT and protein kinase dead mutant of PKM2 Q393K. Tubulin served as internal loading control.

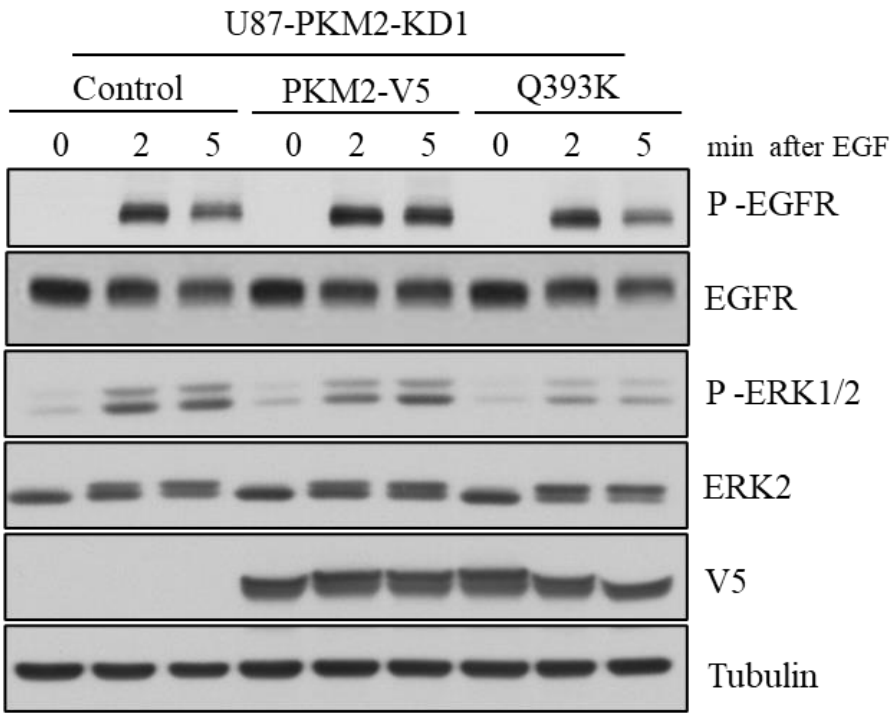


Figure 12.2. Immunoblot for (P-)EGFR, (P-)ERK and PKM2 of U87-CRISPR-construct cells with stable PKM2-knockout that were non-stimulated or stimulated with EGF (20 ng/ml) for 2 and 5 min two days after transient overexpression of PKM2-WT and protein kinase dead mutant of PKM2 Q393K. Tubulin served as internal loading control. The experiment was performed by Dr. Huike Jiao.

4.6.2 PKM2-K433R mutant does not enhance EGFR signalling

As diverse oncogenic stimuli regulate the acetylation at lysine 433 and thus the dimer-tetramer ratio of PKM2, the K433 PKM2 mutants were of special interest for our study.

To examine whether the acetylation at lysine 433 is necessary for PKM2-mediated enhancement of EGFR phosphorylation, we transfected the acetylation mimic PKM2 K433Q mutant and the non-acetylable protein kinase dead mutant PKM2 K433R to U87-CRISPR-construct cells with stable PKM2-knockout. In contrast to the overexpression of wild-type PKM2 or the acetylation-mimic PKM2 K433Q mutant, the non-acetylable protein kinase dead PKM2 K433R mutant did not increase neither P-EGFR- nor P-ERK1/2. Interestingly, the K433Q mutant even increased EGFR phosphorylation further underlining the important promoting role of PKM2 acetylation in EGFR activation. These results are shown in Figure 12.3.

Taken together, the results of the transfection experiments with the protein kinase dead PKM2 mutants Q393K and more with the K433R mutant suggest that these sites and

possibly their posttranslational modifications linked to the protein kinase activity of PKM2 are necessary for PKM2-mediated EGFR phosphorylation.

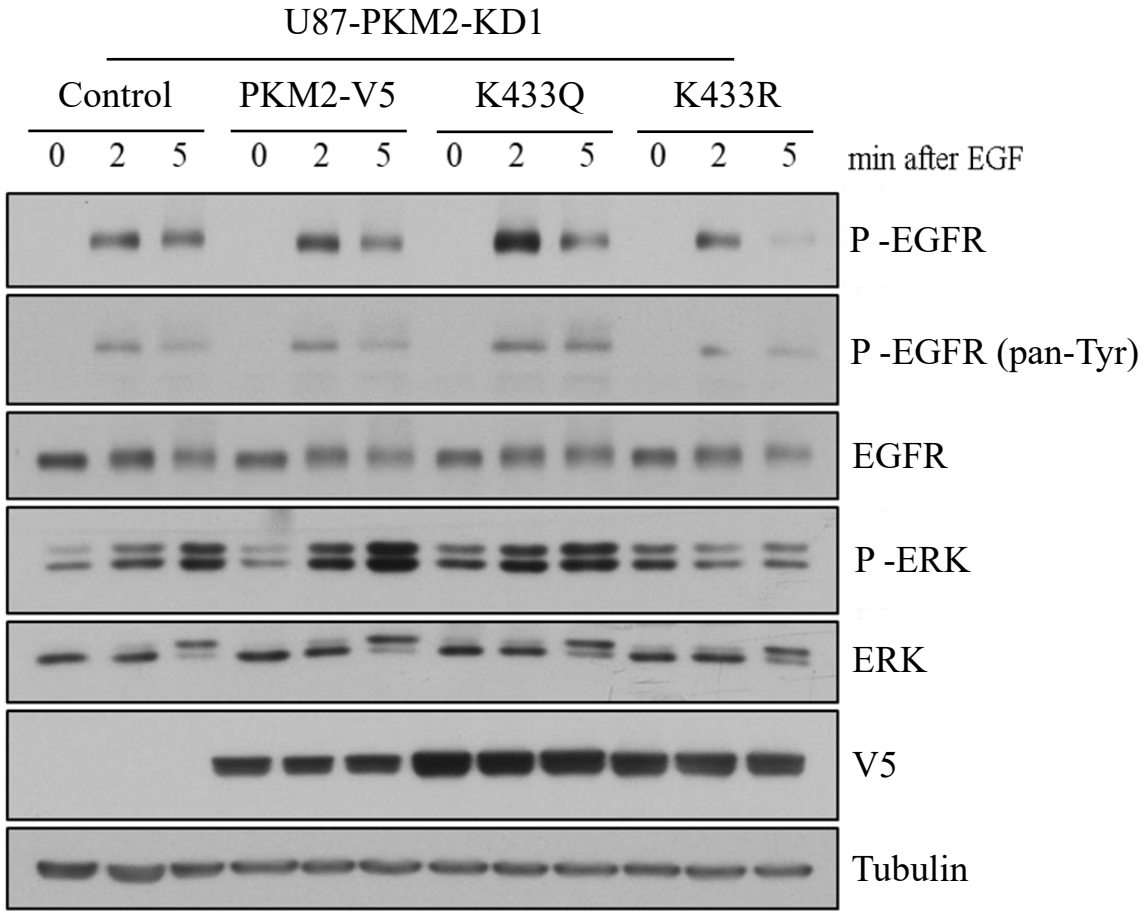


Figure 12.3 Immunoblot for (P-)EGFR, (P-)ERK and PKM2-V5 of U87-CRISPR-construct cells with stable PKM2-knockout that were non-stimulated or stimulated with EGF (20 ng/mL) for 2 min and 5 min two days after transient overexpression of PKM2-WT, acetyl-mimetic mutant of PKM2 K433Q and non-acetylable protein kinase dead mutant of PKM2 K433R. Tubulin served as internal loading control.

4.7 Protein kinase dead mutants of PKM2 (Q393K, K433R) do not enhance tumour cell growth

4.7.1 PKM2-Q393K mutant does not enhance tumour cell growth

To assess the effect of PKM2 mutants on tumour cell growth, the sphere forming assay was performed. In contrast to the overexpression of wild-type PKM2, the PKM2-Q393K mutant did not increase the EGFR-signalling dependent sphere formation (Figure 13.1).

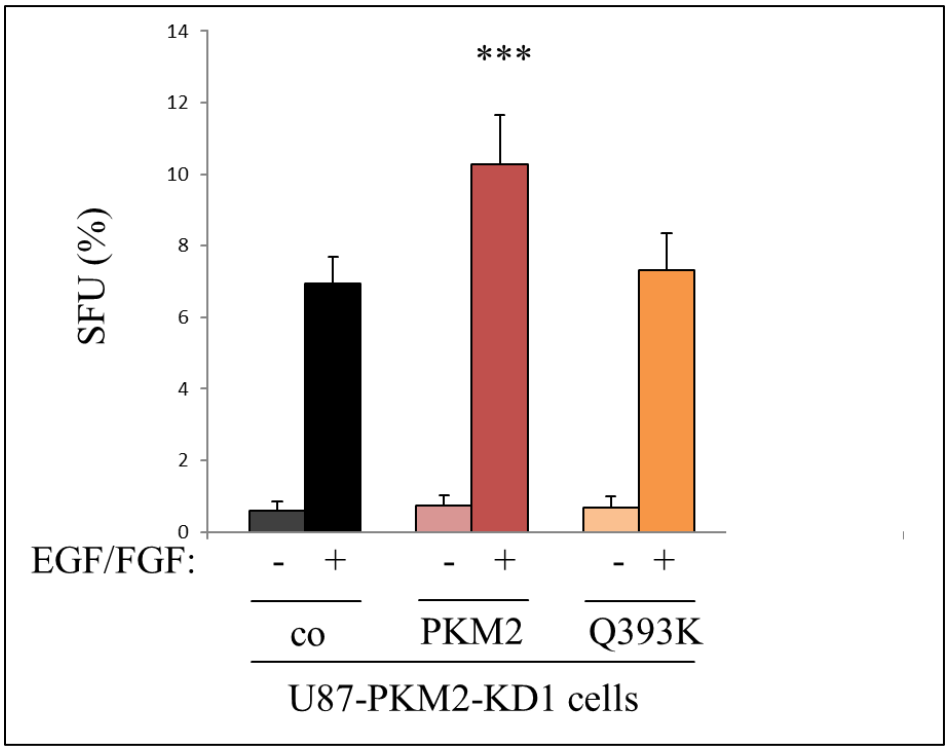


Figure 13.1. U87-PKM2-KD1 cells were cultured as spheroids in B27-supplemented serum-free medium+EGF/FGF (20 ng/mL) one day after transient overexpression of PKM2-WT or PKM2-Q393K. The number of spheroids was quantified (n=6).

4.7.2 PKM2-K433R mutant does not enhance tumour cell growth

Similarly, in contrast to the overexpression of wild-type PKM2 and the acetylation mimic K433Q, the non-acetylable and protein kinase dead PKM2-K433R mutant did not increase the EGFR-signalling dependent sphere formation, too (Figure 13.2).

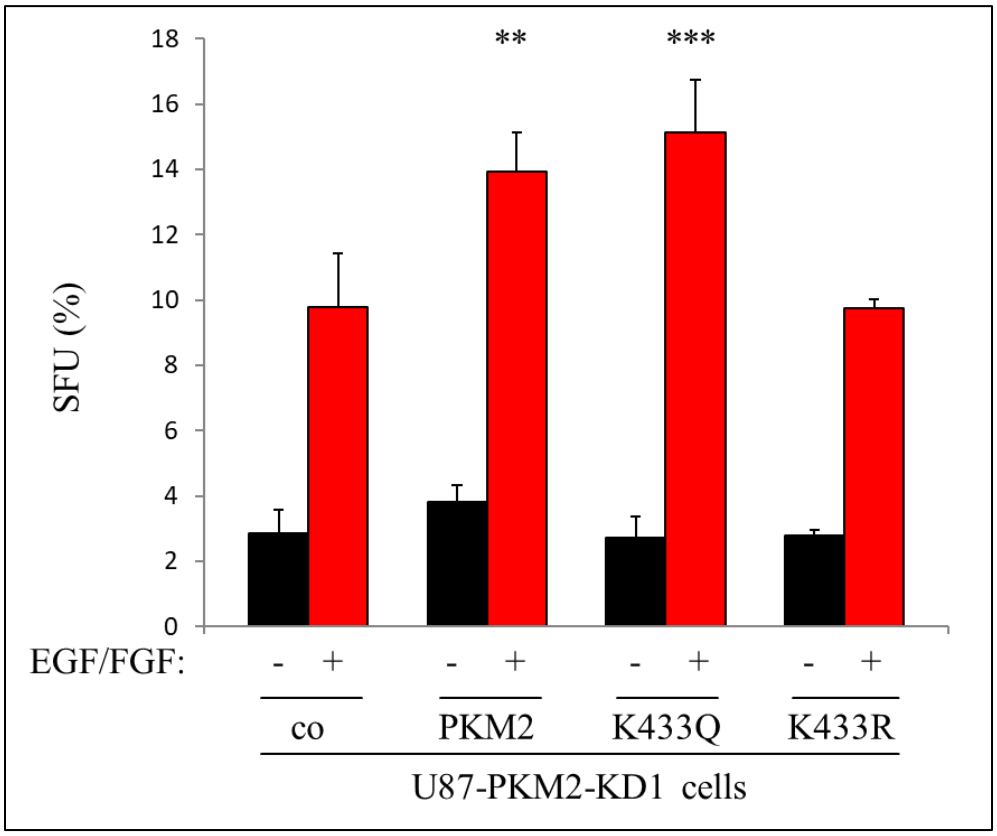


Figure 13.2. U87-PKM2-KD1 cells were cultured as spheroids in B27-supplemented serum-free medium+EGF/FGF (20 ng/mL) one day after transient overexpression of PKM2-WT, PKM2-K433Q or PKM2-K433R. The number of spheroids was quantified (n=6).

4.8 Acetylation at PKM2 residue K433 correlates with the strength of EGFR signalling

As the acetylation status of PKM2 is regulated by various deacetylases, it was of interest whether the treatment with deacetylase inhibitors could influence the EGFR-phosphorylation via PKM2 acetylation status. As previously described, the acetylation status of PKM2's lysine 433 is enhanced after treatment with the deacetylase inhibitor mixture of nicotinamide (NAM) and trichostatin A (TSA) (Lv, Xu et al. 2013).

4.8.1 TSA-induced K433 acetylation of PKM2 correlates with EGFR-phosphorylation

To further examine which of the two deacetylase inhibitors enhance PKM2s acetylation status, and whether thus acetylated PKM2 enhances EGFR phosphorylation as suggested by the acetylation mimic PKM2-K433Q, we treated U87 cells with NAM and/or TSA

deacetylase inhibitors before EGF-stimulation. In contrast to single NAM treatment, TSA supplementation enhanced the EGFR-phosphorylation. Interestingly, in line with its role in inhibiting the deacetylation, TSA increased the signal intensity of a protein detected with an AcK433-PKM2-specific antibody which appears to have a molecular weight slightly below the expected molecular weight of PKM2 (see arrow A in Figure). Moreover, the increase in this minor fraction of acetylated PKM2 seemed to correlate with EGFR-phosphorylation. These results are shown in Figure 14.1.

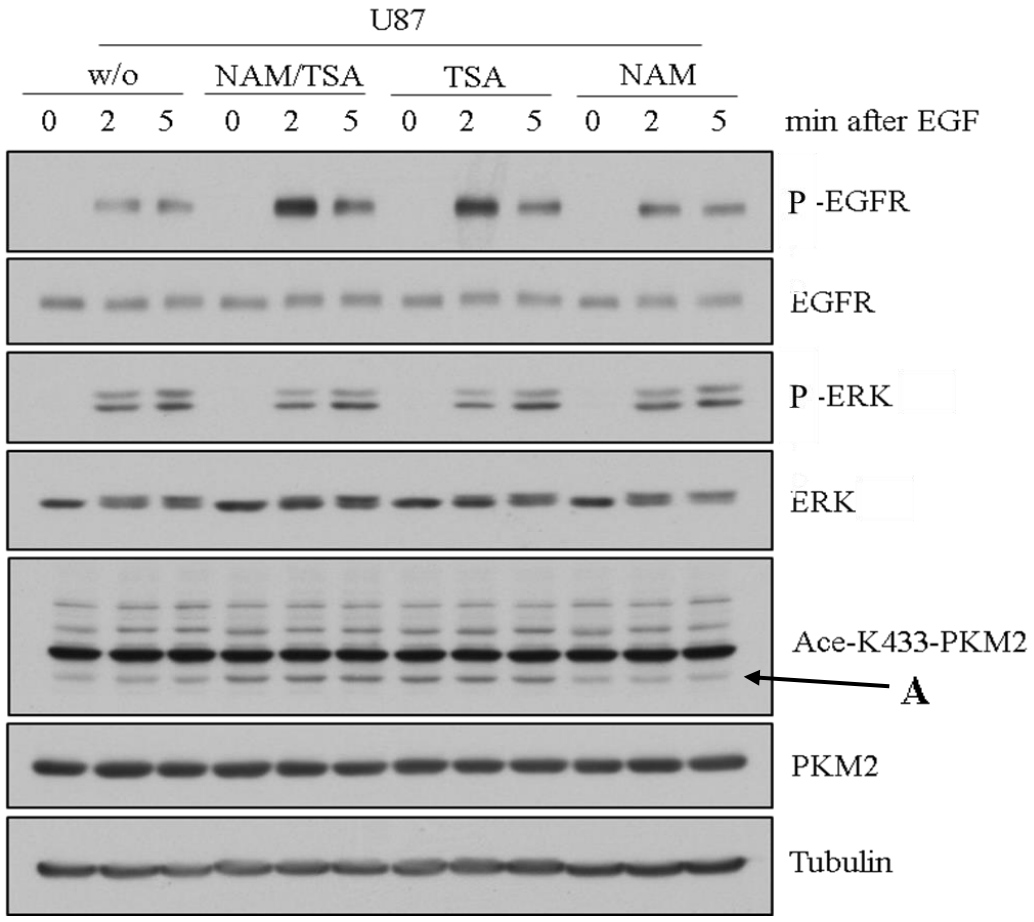


Figure 14.1. Inhibition of deacetylation enhances EGFR phosphorylation. Immunoblot for (P-)EGFR, (P-)ERK, acetylated K433 of PKM2 and PKM2 of U87 cells that were non-stimulated or stimulated with EGF (20 ng/mL) for 2 min and 5 min. Where indicated, cells were pretreated with the deacetylase inhibitors NAM (20 mM) and TSA (10 µM) for 16 hours. Minor fraction of acetylated PKM2 is highlighted with the arrow A. Tubulin served as internal loading control.

4.8.2 TSA-mediated enhancement of EGFR-phosphorylation is PKM2-dependent

The induction of EGFR phosphorylation and the increased PKM2 K433 acetylation upon deacetylase inhibition following TSA suggested a direct link between PKM2 acetylation and EGFR phosphorylation. To further support this finding, we examined whether the enhanced EGFR-phosphorylation directly correlates with PKM2 levels. We therefore overexpressed PKM2 in U87-CRISPR-construct cells with stable PKM2-knockout, treated the cells with TSA and compared their EGFR-phosphorylation status with cells without PKM2-overexpression. Intriguingly, P-EGFR protein levels correlated positively with the levels of a small fraction of proteins detected with an AcK433-PKM2-specific antibody (see arrow B in Figure 14.2), associated with both PKM2-overexpression and TSA treatment. Moreover, higher levels of PKM2 increased TSA-mediated EGFR-phosphorylation, indicating that the interplay between PKM2 and deacetylases affects EGFR-phosphorylation. These results are shown in Figure 14.2.

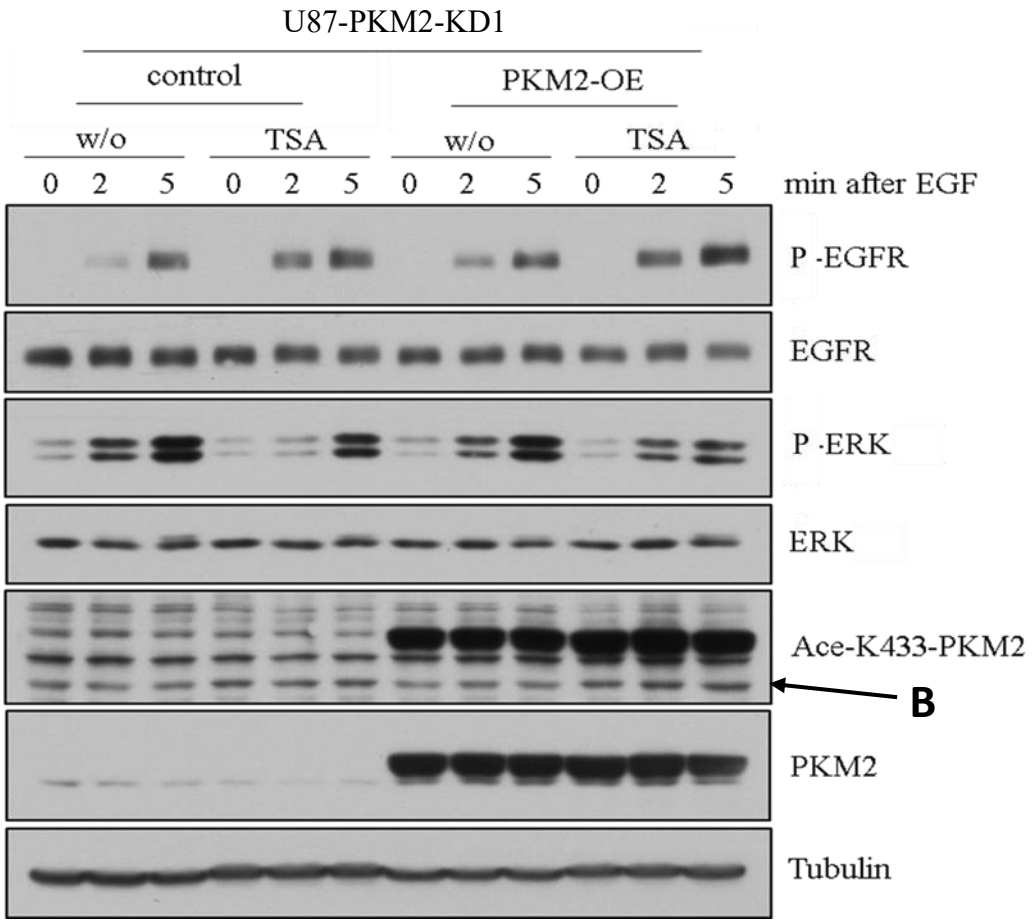


Figure 14.2. Immunoblot for (P-)EGFR, (P-)ERK, acetylated K433 of PKM2 and PKM2 of U87-CRISPR-construct cells with stable PKM2-knockout that were non-stimulated or stimulated with EGF (20 ng/mL) for 2 min and 5 min two days after transient overexpression of PKM2-WT. Where indicated, cells were pretreated with the deacetylase inhibitor TSA (10 µM) for 16 hours. Minor fraction of acetylated PKM2 is highlighted with the arrow B. Tubulin served as internal loading control.

5 Discussion

5.1 The ambiguous role of PKM2 in tumours

This study shows that depletion of PKM2 inhibits (Fig. 9.1) and overexpression of PKM2 promotes tumour cell growth (Fig. 9.2). The tumour growth-promoting effect of PKM2 has been shown in several other *in vitro* studies (Cortés-Cros et al., 2013, Goldberg, Sharp, 2012). However, findings on PKM2 function in tumours differ with respect to their tumour-promoting or tumour-suppressing role. Thus, although both the pyruvate kinase isoform selection for PKM2 over PKM1, and also a PKM2-mediated tumour growth promoting effect during xenograft tumour growth has been observed by some research

groups (Goldberg, Sharp, 2012, Kefas et al., 2010, Ma et al., 2019), a further *in vivo* study suggests that PKM2 deletion accelerates mammary tumour formation, and another one shows that the growth of established xenograft tumours of colon carcinoma cells is unaffected by PKM2 knockdown (Cortés-Cros et al., 2013, Israelsen et al., 2013). The discrepancy of the findings might be explained by the fact that *in vitro* experiments cannot phenocopy the intratumoural heterogeneity and the metabolic tumour microenvironment that exists *in vivo* (Salk et al., 2010) as well as by potential differences in PKM2 function between tumour entities. The *in vivo* findings which suggest that PKM2 is not necessary for tumour growth is backed up by the notion that the PKM1-PKM2 switch does not occur in all malignant tumours (Desai et al., 2014). Nevertheless, tumours might favour PKM2 over PKM1 due to the Warburg effect, which is mediated partly by low PK activity and contributes to tumour cell growth. This hypothesis is in line with the finding that the mammary tumours, whose growth was increased upon PKM2 loss, also showed decreased PKM1 expression (Israelsen et al., 2013). Moreover, inhibition of PK enzymatic activity of PKM2 by phosphotyrosine (Christofk et al., 2008a, Hitosugi et al., 2009), cellular redox state (Anastasiou et al., 2011) or lysine acetylation (Lv et al., 2011, Lv et al., 2013) leads to the Warburg effect and confers tumour growth (Israelsen et al., 2013). This might be the case not only due to the Warburg effect, but also results from the other diverse functions of dimeric PKM2 mentioned above. The role of the tumour growth promoting effect of the lower PK activity in relation to its functions as a protein kinase or a co-transcription factor is still unknown (Lv et al., 2011).

Whether the tumour growth is dependent on lower PK activity of PKM2 might also be determined by the specific genetic profile or the microenvironmental milieu of tumours. This hypothesis is supported by an earlier study, which suggested that PKM1 promotes tumour growth in specific contexts (Morita et al., 2018). Since PKM1 represents a therapeutic target under certain conditions such as paclitaxel-resistant gastric cancer cells (Okazaki et al., 2018), targeting both PKM2 and PKM1 to decrease PK activity and inhibit tumour growth might also be considered (Ma et al., 2019). However, this might be problematic since PK activity is also needed for non-cancer cell growth and survival. According to another hypothesis, non-metabolic regulatory functions of PKM2 could provide the basis for enhanced tumour cell growth and survival. As mentioned above, the activity-regulating mechanisms of PKM2 allow the cancer cells to adapt to different physiological conditions. If the regulation of PK activity is critical for PKM2-dependent

tumour growth, then either PKM2 activation or inhibition could be beneficial to tumour cells in different contexts and dependent on factors such as hypoxia, glucose availability or RTK signalling (Gui et al., 2013). In this case, targeting PKM2 by either PKM2 inhibitors or PKM2 activators (or both) might be an applicable therapeutic approach for cancers with high EGFR signalling activity, such as GBMs. This hypothesis is supported by the notion that EGFR-driven epithelial neoplasia formation and metastasis is driven by the Warburg effect in a Drosophila model (Eichenlaub et al., 2018), and that the expression of PKM2 in GBM also correlates with the EGFR signalling activity (Yang et al., 2012a). In comparison, GBM cells expressing the constitutively active EGFRvIII mutant exhibit a stronger Warburg effect (Furnari et al., 2015). Taken together, these findings strongly indicate that the metabolic constitution of EGFR-driven cancer cells might be more dependent upon PKM2 regulatory mechanisms than the metabolic constitution of other cells. In addition, the role of PKM2 in tumours may differ dependent on whether PKM2 acts through its glycolytic or non-glycolytic function.

5.2 How could PKM2 trigger EGFR signalling?

In this study, both the levels of EGFR phosphorylation (Fig. 7 & 8) and the EGFR-mediated tumour cell growth (Fig. 10) were shown to be enhanced by PKM2. The upregulation of EGFR phosphorylation was independent of PKM2s pyruvate kinase activity, as the PK dead mutant PKM2-K367M also enhances EGFR phosphorylation (Fig. 11). Moreover, we did show that the PKM2 protein kinase dead mutant PKM2-Q393K (Fig. 12.1, 12.2 & 13.1) and the acetylation-deficient mutant PKM2-K433R (Fig. 12.3 & 13.2) do not affect EGFR phosphorylation and tumour cell growth compared to PKM2-WT. This suggests that the protein kinase activity and acetylation of lysine 433 on PKM2 is crucial for upregulation of EGFR signalling and tumour growth.

Based on the two observations above, it will be interesting to investigate the underlying mechanism how PKM2 enhances EGFR activation and how it affects tumour growth. EGFR was reported to interact with PKM2 (Shi et al., 2018). In addition to EGFR, PHD3 was also reported to interact with PKM2, whilst interaction between PHD3 and EGFR promoted the internalization of EGFR, thus suppressing the activation of EGFR signalling (Garvalov et al., 2014, Luo et al., 2011a). Based on these findings, we hypothesized that PKM2 interacts either with EGFR or PHD3, with PHD3 as a negative regulator of EGFR

signalling. Then we asked, whether PKM2 affects the interaction between EGFR and PHD3. This hypothesis was supported by experimental evidence in our laboratory from Dr. Huike Jiao. She found that the interaction between EGFR and PHD3 is suppressed by overexpression of PKM2-WT or the acetylation-mimic mutant PKM2-K433Q (data not shown). Thus, the acetylation of PKM2 on lysine 433 plays a role in EGFR activation being critical for interfering with the interaction between EGFR and PHD3.

Other possible mechanisms of PKM2-dependent enhancement of EGFR signalling have also been proposed in other studies. One possibility is that PKM2 acts as a protein kinase and phosphorylates and regulates either a protein that regulates EGFR activation, or PKM2 phosphorylates EGFR directly similar to the PKM2-mediated phosphorylation of EGFR downstream mediators ERK1/2, Akt1 and Stat3 (Gao et al., 2012, He et al., 2016, Keller et al., 2014). This might well be the case as EGFR was shown to be a potential phosphorylation target of PKM2-SAICAR (Keller et al., 2014), and the SAICAR-insensitive protein kinase dead mutant PKM2-Q393K did not enhance EGFR phosphorylation in our experiments (Fig. 12.1 & 12.2). However, although the protein kinase activity of PKM2 was demonstrated in numerous studies mentioned above, a study by Hosios et al. (2015) challenged this mechanism. This study showed that PKM2 might just regenerate ATP from PEP for other kinases, arguing that this is a plausible explanation for the results of the studies mentioned above. It might still be that the protein kinase activity of PKM2 does not play a role in regulating EGFR phosphorylation, and that the effect on EGFR is diminished just by a decrease of the dimer-tetramer ratio in the case of the PKM2-Q393K mutant. After all, the protein kinase activity is probably interconnected with the dimer formation of PKM2, as the protein kinase activity of the PKM2-SAICAR complex gets inhibited by FBP, while FBP also induces the PKM2 tetramer formation (Anastasiou et al., 2012, Keller et al., 2014). The PKM2 dimer formation enhanced by lysine 433 acetylation however could enhance the protein kinase activity as well. This site is also important for phosphotyrosine-binding. However, the phosphotyrosine-binding ability of the 433 site does not seem to be important for EGFR phosphorylation, as the overexpression of the phosphotyrosine-binding deficient PKM2-K433E mutant (Christofk et al., 2008b) did show just the same effect on EGFR phosphorylation as PKM2-WT in our preliminary results (data not shown). As the acetylation of K433 is stimulated by a diversity of different oncogenic stimuli mentioned above including EGF stimulation (Lv et al., 2013, Zhang et al., 2019), the acetylation of

this site might be a starting point of a positive feedback-loop mechanism illustrated in the model below (Fig. 15). Based on the findings in this study, we hypothesize that upon acetylation of PKM2 at site K433, the PKM2 dimer directly or indirectly increases EGFR-phosphorylation, which in turn enhances both the PKM2 dimer-tetramer ratio and PKM2 expression.

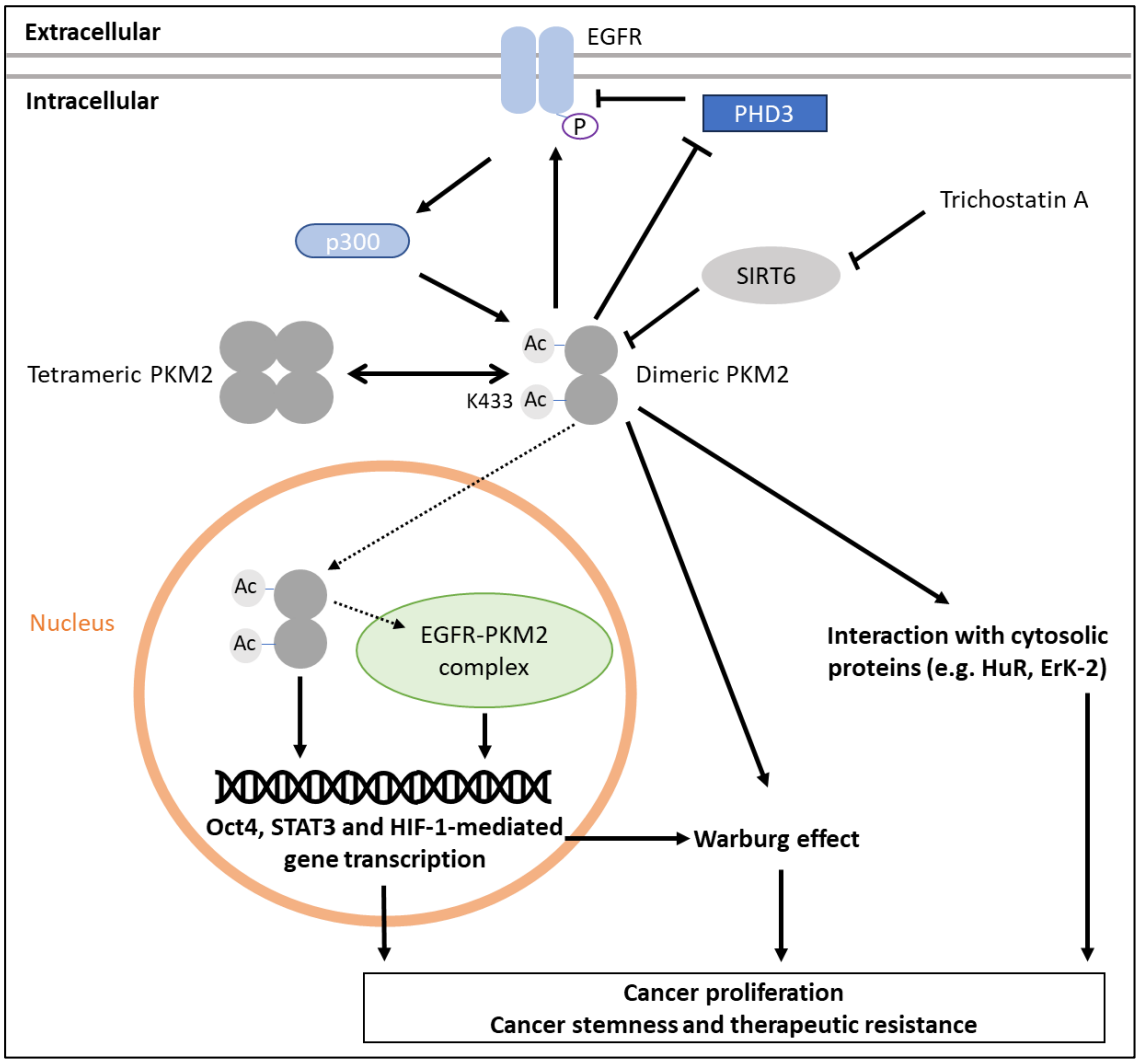


Figure 15. EGFR signalling enhances both the PKM2 dimer-tetramer ratio and the PKM2 expression. Furthermore, upon EGF stimulation, PKM2 gets acetylated at K433 by p300, which favours dimer formation (Prakasam et al., 2018). In turn, both the increased levels of nuclear and cytoplasmic dimeric PKM2 and the nuclear complex of PKM2 and EGFR promote tumour growth and the CSC phenotype, which on the other hand fuels EGFR-driven cancer formation, metastasis and therapeutic resistance (Kim et al., 2009, Morfouace et al., 2014, Shi et al., 2018, Yang et al., 2011). SIRT6 was shown to deacetylate K433 (Bhardwaj, Das, 2016) and Trichostatin A (TSA) is the most potent known SIRT6 inhibitor (You, Steegborn, 2018). Our results suggest that increased levels of Ac-K433-PKM2 may enhance EGFR-phosphorylation upon EGF stimulation, and that PKM2 might enhance EGFR-phosphorylation directly through its protein kinase activity or through interaction with PHD3.

There might be further possible activation processes of the proposed feedback loop. The PKM2 dimer might enhance its own secretion and activate the EGFR signalling pathway in an autocrine manner (Hsu et al., 2016, Wei et al., 2017, Wan et al., 2019).

Furthermore, EGFR signalling activates the NADPH-oxidase, which induces the production of various instable ROS (Bae et al., 1997). This might lead to enhanced cysteine 358 oxidation of PKM2, which induces the dimerization of PKM2 (Anastasiou et al., 2011). However, our preliminary results did show that the overexpression of the oxidation-resistant mutant C358S of PKM2 does not decrease the levels of EGFR phosphorylation in comparison to PKM2-WT (data not shown). Further studies are needed to examine the mechanism by which PKM2 enhances EGFR-phosphorylation. As the protein kinase dead mutant PKM2 Q393K does not enhance EGFR-phosphorylation (Fig. 12.1 & 12.2), the acetylated PKM2 dimer might need its protein kinase activity to either phosphorylate EGFR directly, or indirectly phosphorylate other proteins which in turn would lead to EGFR-phosphorylation. Another possible mechanism for enhancement of EGFR-phosphorylation is the above mentioned interaction of PKM2 with PHD3. Coimmunoprecipitation assays might be able to show if PKM2 binds to EGFR in order to enhance its activation, and if so, which posttranslational modifications and proteins are engaged in this mechanism. In fact, preliminary results of Dr. Huike Jiao`s coimmunoprecipitation assay hint that PKM2 binds to EGFR, that acetylation on lysine 433 is required for its interaction with EGFR and that PHD3 enhances the interaction between EGFR and PKM2 (data not shown).

However, as only a small fraction of acetylated PKM2 may enhance EGFR-phosphorylation (fraction A and B in Fig. 14.1 and 14.2 respectively), it might be difficult to detect it in some experiments. Moreover, further experiments with direct and indirect PKM2-inhibitors are needed to rule out the possibility, that TSA does not enhance EGFR-phosphorylation via PKM2, but instead independently enhances both PKM2-K433 acetylation and EGFR-phosphorylation.

5.3 The PKM2-EGFR axis is a potential therapeutic target

The experiments in this study strengthened the notion that PKM2 is an important determinant of tumour cell growth in EGFR-driven cancer cells (Fig. 10). As EGFR

alterations are typically the sole and by far the most dominant RTK lesions in the majority of glioblastoma multiforme (Brennan et al., 2013, Furnari et al., 2015), the EGFR signalling is an attractive therapeutic target. This remains true despite disappointing clinical studies up to date (Le Rhun et al., 2019). Both EGFR and PKM2 are upregulated in GBM, with dimeric nuclear PKM2 correlating with EGFR activation (Li et al., 2016, Yang et al., 2012c).

Targeting the PTMs of PKM2 constitutes a promising strategy for cancer treatment (Prakasam et al., 2018). This is because PKM2 exhibits a diversity of cancer-promoting effects in PTM-related manners. Specifically, both the phosphorylated and acetylated PKM2 enhances the PKM2 gene transcription in a positive feedback loop pattern upon hypoxia and EGFR activation (Luo et al., 2011b, Lv et al., 2013, Yang et al., 2012c). Our study provides further hint for the notion that the acetylation of the PKM2-specific lysine 433 might represent a promising target, as the *in vitro* treatment with the deacetylase inhibitor trichostatin A (TSA) enhanced the PKM2-mediated EGFR phosphorylation (Fig. 14.1). Other studies have shown that both the tumour suppressive deacetylase SIRT6 and the acetyltransferase p300, which both represent potential therapeutic targets in cancer treatment (van Meter et al., 2011, Giotopoulos et al., 2016), are regulators of K433 acetylation (Lv et al., 2013). Moreover, analysis of hepatocellular carcinoma samples revealed that the levels of acetylation at PKM2 K433 get elevated with increasing grades of liver carcinoma, whereas the SIRT6 levels decline (Bhardwaj, Das, 2016). If the effect of TSA is mediated by SIRT6, which regulates the deacetylation at lysine 433, SIRT6 might emerge as a protein that regulates PKM2 function both directly and indirectly. This is because loss of SIRT6 inhibits PARP1 (Mao et al., 2011), which in turn activates both nuclear retention and nuclear functions of PKM2 in EGFR-driven cancer cells (Li et al., 2016). In conclusion, we suggest that SIRT6 activators could constitute a promising approach for targeting the PKM2-EGFR axis in cancer cells. Interestingly, SIRT6 activators already have been proposed for the treatment of cancer, metabolic syndrome and insulin-resistant diabetes elsewhere (Tasselli et al., 2017, van Meter et al., 2011), and the first synthetic SIRT6 activator has been developed recently (Iachettini et al., 2018).

However, the question remains whether PKM2-mediated EGFR activation results from SIRT6 inhibition, or from inhibition of a different deacetylase, as the effect of TSA might also be indirect. This may apply as TSA, on the one hand, is the most potent known SIRT6

inhibitor with no proven effect on other sirtuins, yet on the other hand was shown to inhibit class I and II histone deacetylases (You, Steegborn, 2018).

Also, TSA may further modify the activity of oncogenic tyrosine kinases, such as the Src kinase (Hirsch et al., 2006), which are involved in the regulation of both EGFR and PKM2 phosphorylation (Jahns et al., 2012).

The other deacetylase inhibitor nicotinamide (NAM) used in our experiments is a pan-sirtuin inhibitor (Cen et al., 2011). However, NAM did not show any effect on the acetylation of PKM2 (Fig. 14.1). As NAM leads to a weaker SIRT6 inhibition compared to TSA (Wood et al., 2018, You, Steegborn, 2018), the reason for this might just be an insufficient inhibition of SIRT6. However, this remains speculative. The fact that several deacetylase inhibitors and activators have been developed for anti-cancer treatments and are being tested in pre-clinical and clinical studies (Dai et al., 2018, Eckschlager et al., 2017, Nadeem Abbas et al., 2019) demonstrates the importance of investigating mechanisms behind how TSA or SAHA enhance EGFR phosphorylation (Zhou et al., 2006). It might involve a crucial mechanism that makes those agents less effective for the treatment of EGFR-driven cancer, or even promote tumorigenesis as an adverse effect. Our working model proposes such a mechanism (Fig. 15).

Whatever the mechanism of action might be, it is intriguing that only a small fraction of acetylated PKM2 (fractions A and B in Fig. 14.1 & 14.2 respectively) correlated with the activation of EGFR. The lower molecular weight of this fraction might be caused by cleavage of PKM2. Interestingly, the inhibition of this small fraction might have a large impact on EGFR-driven cancer cells, if this PKM2 fraction is indeed Ac-K433-PKM2, which is responsible for the enhanced EGFR activation seen in our experimental results. However, this new and unexpected finding needs further investigation before we can make any robust conclusions.

Interestingly, there are also other lines of evidence that make EGFR and PKM2 look attractive as part of a combinational therapeutic strategy. It was shown that PKM2 is a therapeutic target in GBM that has the potential for exhibiting minimal toxicity to the brain. Moreover, PKM2 inhibition shows effectiveness against glioblastoma CSCs resistant to the standard therapies (Kefas et al., 2010). As mentioned above, the targeting of CSCs is of enormous relevance for the development of therapies aiming to completely and lasting eliminate cancer. It is a viable strategy of inhibiting CSC formation via

targeting either PKM2, EGFR or their interaction, as both the EGF-induced nuclear PKM2 dimer and the nuclear complex of PKM2 and EGFR is involved in the transcription of CSC-related genes (Kim et al., 2009, Morfouace et al., 2014, Shi et al., 2018, Vermeulen et al., 2010, Yang et al., 2011). Another important point, which has already been illustrated above, is that targeting the PKM2-mediated Warburg effect should be more promising in cancer cells with higher EGFR activation, e.g. the classical molecular subgroup of GBM.

Lastly, the interaction between PKM2 and EGFR signalling involves positive feedback mechanisms. This study comprises a working model of a new positive feedback loop. The activation of positive feedback mechanisms can lead to dramatic shifts in cellular responses (Santos et al., 2007). Positive feedback increases the sensitivity of the system to signalling inputs like EGF by amplifying the stimulus (Lemmon, Schlessinger, 2010), revealing properties essential for EGFR signalling regulation and posing an attractive mechanism as potential therapeutic target.

The proposed positive feedback loop of our working model gains even further significance, as it is not the only positive feedback mechanism which might get stimulated through the PKM2-EGFR axis. The interplay between the activation of EGFRs downstream mediator ERK1/2 and PKM2 mentioned above constitutes a positive feedback mechanism as well (Keller et al., 2014).

Through disrupting the feedback mechanism illustrated in our working model one might diminish the growth promoting effects of EGFR signalling, PKM2 and their interplay. According to these considerations, several drugs might work through disrupting the positive feedback loop proposed in our working model. First, the SIRT6 activators might work through this mechanism. SIRT6 activators may decrease NF-kB-driven PKM2 expression (Kawahara et al., 2009, Yang et al., 2012a), and counteract the epigenetic modifications induced by nuclear PKM2. This could occur because SIRT6 deacetylates histone H3, whose acetylation is mediated by the protein kinase function of nuclear PKM2 (Michishita et al., 2008, Yang et al., 2012b). SIRT6 thus blocks PKM2-induced gene expression such as Myc and HIF-1 (Zhong et al., 2010), ultimately opposing the Warburg effect and targeting the metabolism of cancer cells. Second, besides SIRT6, ß-elemene might disrupt the feedback mechanism between PKM2 and EGFR as well. ß-elemene is a compound that inhibits the development of breast cancer metastasis in mice via blocking the dimerization and nuclear translocation of PKM2. Interestingly,

ß-elemene also downregulates the EGFR signalling pathway (Pan et al., 2019). Finally, the PKM2-inhibitor shikonin might also be able to disrupt the feedback mechanism between PKM2 and EGFR. Shikonin may inhibit cancer cell glycolysis (Chen et al., 2011), migration and invasion in a wide range of tumours, including GBM (Matias et al., 2017, Wang et al., 2019). Moreover, shikonin is a drug that synergistically promotes both NSCLC and GBM tumour cell death in combination with erlotinib (Li et al., 2018, Zhao et al., 2015).

There are many mechanisms of how cancer becomes resistant to single TKI inhibition. In line with preclinical studies observing that combination therapy is a promising approach in cancer therapy not only to overcome resistance but also to reduce the dosages of both agents to minimize adverse effects (Yang, Tam, 2018), we propose to target both EGFR and PKM2s acetylation in EGFR-driven GBM. This combination therapy may lead to synergistic tumour growth inhibition via targeting the therapy-resistant cancer stem cells.

Targeting both EGFR and PKM2 acetylation might be accomplished by using TKIs together with the newly developed synthetic SIRT6-activators. The SIRT6-activators might affect the characteristic metabolism of GBM cancer cells through the deacetylation of PKM2, leading to impairment of PKM2-dependent signalling of wild-type EGFR and impeding various other cancer-driving mechanisms mentioned above. SIRT6 might even effect constitutively active mutant EGFRvIII signalling in GBM. This is because suppression of nuclear function of PKM2 through the inhibition of SIRT6-regulated PARP suppresses tumour growth of EGFRvIII expressing glioblastoma multiforme cells (Li et al., 2016). Through its multiple points of interference and disruption of cancer driving positive feedback loops in particular the simultaneous use of SIRT6-activators and TKIs might help overcome resistance in EGFR-driven glioblastoma, warranting further studies of this combination.

6 Summary

Despite promising progress in treating various cancers with targeted therapy against the epidermal growth factor receptor (EGFR), its therapeutic effect in glioblastoma multiforme (GBM) is still poor. EGFR signalling has been shown to be susceptible to regulation by pyruvate kinase M2 (PKM2). Moreover, EGFR was revealed as a potential target of the PKM2 protein kinase. This suggests that PKM2 may interact with EGFR and EGFR-binding proteins.

The aim of this work is to elucidate whether and by which molecular mechanisms PKM2 may regulate the activation of EGFR signalling and tumour growth. For this purpose, we generated stable PKM2 loss-of-function GBM cell lines by CRISPR-mediated knockout and performed rescue experiments *in vitro* with various PKM2 overexpression constructs. Western blots and sphere formation assays revealed that PKM2 overexpression enhances both EGFR phosphorylation and tumour cell growth upon EGF stimulation. To check whether the PKM2-induced tumour cell growth is mediated through EGFR signalling, we inhibited EGFR activity with its specific inhibitor erlotinib. To determine which post-translational modifications (PTMs) and features of PKM2 are essential for its function on EGFR, we generated a variety of PKM2 mutants using site-directed mutagenesis, and investigated their effect on EGFR activation. The results show that the PHD3 hydroxylation site on PKM2 (proline 403 and 408) and acetylation of PKM2 on lysine 433 is required for enhancing EGFR activation. This indicates that these modifications, known to be associated with the protein kinase activity of PKM2, may promote EGFR activation and tumour growth. To test this hypothesis, we investigated whether the deacetylase inhibitors trichostatin A (TSA) or nicotinamide (NAM) cause a PKM2-mediated upregulation of EGFR signalling. Unexpectedly, we found that a small fraction of acetylated PKM2 correlated with the TSA-induced activation of EGFR.

The results of this work strengthen the notion that PKM2 is an important determinant of tumour growth in EGFR-driven cancer cells, and they suggest that PKM2 promotes EGFR activation via a positive feedback-loop mechanism. From a therapeutic point of view, our results highlight the PKM2 lysine 433 acetylation as a potential therapeutic target, indicating that deacetylase activators might in part act through the PKM2-EGFR axis in cancer cells.

7 Zusammenfassung

Trotz klinischer Wirksamkeit der gezielten Therapie gegen die Rezeptortyrosinkinase EGFR zur Behandlung verschiedener Krebsarten, ist die Wirkung dieses Ansatzes bei Glioblastoma multiforme (GBM) bislang begrenzt. Der EGFR-Signalweg ist durch das potenziell therapeutische Target Pyruvatkinase M2 (PKM2) beeinflussbar. Auch wurde gezeigt, dass EGFR ein potenzielles Ziel der Proteinkinaseaktivität von PKM2 darstellt, sodass PKM2 mit EGFR und an EGFR bindenden Proteinen interagieren könnte.

Ziel dieser Arbeit ist zu klären, ob und über welche molekularen Mechanismen PKM2 die Aktivierung des EGFR-Signalwegs sowie Tumorwachstum regulieren kann. Hierfür entwickelten wir mittels CRISPR-mediiertem Knockout GBM-Zelllinien mit Funktionsverlust von PKM2 und führten *in vitro* Rescue-Experimente mittels Überexpression von PKM2-Konstrukten durch. Western Blots und Sphere Formation Assays zeigten, dass Überexpression von PKM2 sowohl EGFR-Phosphorylierung als auch das Tumorwachstum nach EGF-Stimulation fördert. Um zu überprüfen, ob das PKM2-induzierte Tumorwachstum vom EGFR-Signalweg mediiert wird, hemmten wir die EGFR-Aktivität mit dem spezifischen EGFR-Inhibitor Erlotinib. Zur Identifizierung posttranslationaler Modifikationen von PKM2, welche die EGFR-Funktion beeinflussen, generierten wir PKM2-Mutanten mittels gezielter Mutagenese und untersuchten deren Einfluss auf die EGFR-Aktivierung. Unsere Ergebnisse zeigen die Notwendigkeit der PHD3-Hydroxylierungsseite Prolin 403 und 408 sowie der Acetylierung von Lysin 433 in PKM2 für gesteigerte EGFR-Aktivität. Dies lässt vermuten, dass die mit Proteinkinase-Aktivität relatierten Modifikationen von PKM2 EGFR-Aktivierung und Tumorwachstum fördern können. Um dies zu testen untersuchten wir, ob die Deacetylase-Inhibitoren Trichostatin A (TSA) bzw. Nicotinamide (NAM) zu einer PKM2-mediierten Hochregulierung des EGFR-Signalwegs führen. Tatsächlich korrelierte eine kleine Fraktion von acetyliertem PKM2 mit TSA-induzierter Aktivierung von EGFR.

Diese Ergebnisse unterstreichen die Rolle von PKM2 beim Tumorwachstum EGFR-abhängiger Krebszellen und lassen geförderte EGFR-Aktivierung über eine positive Rückkopplungsschleife vermuten. Aus therapeutischer Sicht wird die Bedeutung des acetylierten Lysins 433 von PKM2 als ein potenziell therapeutisches Target betont, sowie die mögliche Wachstumshemmung von Krebszellen durch Deacetylasen-Aktivatoren über die PKM2-EGFR-Achse.

8 Acronyms and abbreviations

Ac-	acetylated
AcetylCoA	acetyl coenzyme A
Akt	protein kinase B
AMP	5' adenosine monophosphate
AMPK	AMP kinase
APS	ammonium persulfate
ATP	adenosine triphosphate
BLAST	Basic Local Alignment Search Tool
BSA	bovine serum albumin
Cas9	CRISPR-associated protein 9
CBP	CREB-binding protein
CD	cluster of differentiation
Cl	chloride
co	control
CRISPR	clustered regularly interspaced short palindromic repeats
CSC	cancer stem-like cells
dH2O	aqua ad iniectabilia
DMEM	Dulbecco´s modified eagle medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide
E. coli	Escherichia coli
ECL	enhanced chemiluminescence
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
EGFRvIII	EGFR variant III
ER	endoplasmatic reticulum
ERK	extracellular-signal regulated kinase
FBP	fructose-1,6-biphosphate
FBS	fetal bovine serum
FGF	fibroblast growth factor
FGFR	fibroblast growth factor receptor

fig.	figure
GBM	glioblastoma multiforme
GCN5	general control non-depressible 5
Gly	glycine
h	hour
HCl	hydrochloric acid
HDAC	histone deacetylase
HEPES	4-2-hydroxyethyl-1-piperazineethanesulfonic acid
HIF	hypoxia inducible factor
HPV	human papilloma virus
HRP	horseradish peroxidase
HSP90	heat-shock protein 90
HuR	human antigen R
IDH	isocitrate dehydrogenase enzyme
IL	interleukin
KD	knockout depletion
KO	knockout
LB	lysogeny broth
LDH	lactate dehydrogenase enzyme
MET	receptor tyrosine kinase Met
MGMT	O^6-methylguanine DNA methyltransferase
min	minute
mL	millilitre
MLC2	Myosin light chain 2
mA	miliampere
mM	milimolar
mTOR	mammalian target of rapamycin
NaCl	sodium chloride
NADPH	nicotinamide adenine dinucleotide phosphate
NAM	nicotinamide
NF-kB	nuclear factor 'kappa-light-chain-enhancer' of activated B-cells

ng	nanogram
NSCLC	non-small cell lung cancer
Oct4	octamer-binding transcription factor 4
OD	optical density
OE	overexpression
PARP	poly ADP ribose polymerase
PBS	phosphate-buffered saline
PCAF	p300/CBP-associated factor
PCR	polymerase chain reaction
PDK	pyruvate dehydrogenase kinase
PEP	phosphoenolpyruvate
PHD	prolyl hydroxylase
PI3K	phosphoinositide 3-kinases
PK	pyruvate kinase
PTEN	phosphatase and tensin homolog
PTM	posttranslational modification
PVDF	polyvinylidene fluoride
Rb	Retinoblastoma tumour suppressor gene/protein
RNA	ribonucleic acid
ROS	reactive oxygen species
rpm	revolutions per minute
RTK	receptor tyrosine kinase
SAHA	suberoylanilide hydroxamic acid
SAICAR	phosphoribosylaminoimidazolesuccinocarboxamide
SDS-PAGE	sodium dodecylsulfate polyacrylamide gel electrophoresis
sec	second
SFA	sphere formation assay
SFU	sphere-forming units
sgRNA	single guide RNA
siRNA	small interfering RNA
SIRT	silent mating type information regulation 2 homolog
SNAP-23	synaptosome associated protein 23
SNARE	soluble NSF attachment receptor
Stat	signal transducer and activator of transcription

TCA	tricarboxylic acid
TEMED	tetramethylethylendiamine
TKI	tyrosine kinase inhibitor
TSA	trichostatin A
TSM	tumour sphere medium
V	Volt
w/o	without
WT	wild-type

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I hereby declare that I have completed the present work independently and without unauthorised assistance or use of other than stated aids. All text passages quoted or paraphrased from published or unpublished writings, and all information based on oral statements, have been identified as such. In the research I conducted and mentioned in the dissertation, I have adhered to the principles of good scientific practice as laid down in the 'Satzung der Justus-Liebig-Universität Gießen zur Sicherung guter wissenschaftlicher Praxis', and have complied with ethical, data protection, and animal welfare principles. I assure that no third party has received direct or indirect monetary benefits for work related to the content of the submitted dissertation; otherwise, I have stated them below. I further assure that the submitted thesis has not been presented in the same or a similar form to any other examination authority, be it domestically or abroad, for the purpose of a doctorate or any other examination procedure. All material taken from other sources and persons, which was used or directly referenced in the work has been identified as such. In particular, all individuals who were directly or indirectly involved in the composition of the present work have been named. I agree to have my dissertation verified by a plagiarism detection software or an internet-based software program.

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