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Protein kinase A complexes link metabolic pathways with cancer proliferation

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This final research report should summarize my research stay at the Lab of JS. Gutkind at the Cancer Moores Research Center in La Jolla from the **25th of march until 25th of June** 2019. The time I was working in the Lab of Dr. Gutkind I contributed, besides my main project, to several side-projects of his research group. In this final report I am going to summarize only results from my main project that I took over in the lab.

TITLE OF RESEARCH PROJECT

Protein Kinase A complexes link metabolic pathways with cancer proliferation

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1. Abstract

G protein coupled receptors (GPCR) sense and convert the vast array of extracellular input signals and transmit information through intracellular signaling circuits [1]. Deregulation of receptor-controlled kinase pathways can contribute to the etiology and progression of cancer [2]. One example are activating mutations in the Gαs protein such as R201C [2, 3] leading to hyperactivation of adenylate cyclase and constitutive downstream activation of the cAMP-dependent protein kinase A (PKA) [4]. In order to get a more detailed understanding of the molecular basis of deregulated PKA signaling, we use system-biology-based approaches in order to generate whole kinase interaction networks. This approach could unveil key modulators, responsible for imbalanced cancer cell proliferation and therefore to the identification of proliferation-relevant substrates and druggable effector proteins for therapeutic intervention. To do so, we determined the phospho-proteomic composition of macromolecular PKA complexes [5] from a variety of colorectal cancer cells and human glioblastoma biopsies. Following affinity isolation of endogenous macromolecular PKA complexes we implemented a subtractive phospho-proteomic approach to decipher cell-type specific links to proliferative signaling. Using gene annotations and enrichment analyses we determined a quantitative core PKA protein-protein-interaction network. By the use of this strategy we revealed possible connections of PKA to key modulators of metabolic pathways, which are central components of glycolysis, pyrimidine, and lactate metabolism [6]. We selected interactors and key metabolic molecules that are upregulated in specific cancers and confirmed their PKA-dependent phosphorylation. One major hit is the 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 3 (PFKFB3), which plays a key role in glycolysis and in coordinating cell cycle [7]. We initiated a pharmacology approach to antagonize colon cancer cell proliferation by selective perturbation of PFKFB3 *in vitro* and *in vivo*. Besides that, we use transcriptomic analyses to identify other functional elements of the genome which might be affected by PFKFB3.

2. SCIENTIFIC BACKGROUND

2.1. G-protein-coupled-receptors

Cells are undergoing constitutive adaption to environmental changes. Therefore, the ability to receive, to convert and to transport external stimuli throughout the cell is a basic and highly regulated biological process [8, 9]. The cell normally responses to signals in form of chemicals, such as second messengers (cAMP), neurotransmitter (acetylcholine), hormones (thyroxine-binding globulin) or growth factors (Fibroblast Growth Factor) [10]. But besides that, proteins must sense and transduce signals between central signaling checkpoints. For receiving external stimuli, cells have proteins called receptors. One of the most abundant groups of receptors are transmembrane proteins. These proteins are connecting the extra and intracellular space in order to transmit information from one side of the membrane to the other. The part located in the external medium has a receptor binding sites for special ligands such as epidermal growth factors (EGFs) or GPCR ligands [11]. In turn, the cytoplasmic part of the receptor has the task to recruit and subsequently activate intracellular proteins such as kinases, anchoring proteins or G-proteins [12].

One main group of receptors are so called G-protein-coupled receptor (GPCRs). They consist of seven transmembrane-spanning segments, leading to an N-terminal tail showing towards the extracellular part and an intracellular C-terminal tail. In its inactive (GDP bound) state, the heterotrimeric G protein (consisting of an alpha, beta and gamma subunit) is anchored next to the receptor [12]. Upon binding of a ligand to the receptor it undergoes a conformational change which triggers the physically exchange of GDP to GTP and subsequently the dissociation of the beta and gamma subunit [13]. This GTP-bound alpha subunit is than capable of interacting with other membrane proteins located in the membrane such as adenylate cyclase (AC). This enzyme is responsible for converting adenosine-triphosphate (ATP) to cyclic-adenosine-monophosphate (cAMP) [14]. This second messenger is of high relevance because of its role in activating protein kinase A (PKA) which is involved in a huge variety of phosphorylation events in the cell.

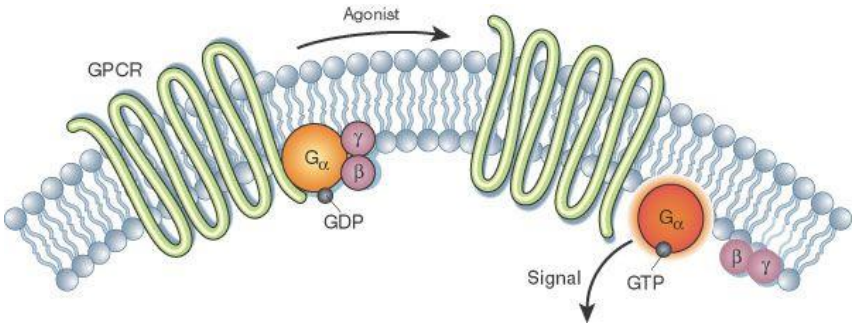

Figure 1| Schematic representation of the activation mechanism of a GPCR: In the absence of an external ligand, the G-alpha is GDP loaded and interacting with the G beta and gamma subunit and the G-protein-coupled receptor. Upon receptor stimulation by a ligand the receptor changes its structure, leading to the dissociation of the G alpha from the receptor and GTP is exchanged for the bound GDP, which leads to G alpha activation. This active G alpha subunit can than activate other proteins in the cell. Picture adopted from Li et. al. [15]

GPCRs play an outstanding role in the human pathophysiology and therefore in progression and formation of different diseases such as diabetes, Alzheimer's disease or cancer [16]. Therefore, it is no surprise, that GPCRs are the most intensively studied drug targets. Nearly 30% of all human cancers are harboring from mutations in GPCRs or G proteins which illustrate the need for mechanistic understanding on the molecular level [2]. One activating mutation is located at position R201 and is suggested to be oncogenic in such a way, that it leads to the inhibition of GTP hydrolyses of the G-protein. The result is a constitutive active adenylate cyclase. Mutations such as R201C or R201H can thereby influence cell growth and cancerogenesis [17]. Furthermore, recent studies showed that GNAS R201C mutation can be found in around 9 % of colorectal cancers [18].

2.2. Protein-kinase A signalling

As mentioned before, second messengers can convert and amplify extracellular signals by activating protein kinases. Adenylate cyclase (AC) for example converts adenosine triphosphate (ATP) into the second messenger cyclic-adenosine-monophosphate (cAMP) which in turn triggers the activation of the cAMP-dependent protein kinase A (PKA). This kinase can then lead to several phosphorylation events.

PKA exists as a tetrameric holoenzyme consisting of two catalytic subunit (C) and two regulatory subunit (R) dimers. Upon binding of cAMP to the regulatory subunit of PKA, the two catalytic subunits are released from the holo-complex, leading to its activation [19]. PKA can contribute to a variety of cellular processes such as modulation of cell differentiation and proliferation [20]. Hereby, diverse scaffolding proteins play important roles by interlinking receptors (GPCRs) and intracellular effectors such as PKA. A collection of scaffolds such as A-kinase anchoring proteins (AKAPs) play key roles in redirecting the information flow [21]. The group of AKAPs can bind to the regulatory subunits of PKA via amphipathic helix motifs and recruit this kinase to different compartments in the cell which leads to subcellular compartmentalized signaling [22]. This is one of the most important regulatory mechanisms in the cell because it facilitates local protein phosphorylation. Deregulation in kinase pathways such as cAMP/PKA can therefore lead to the etiology and progression of cancer.

2.3. Protein-kinase A signalling in cancer

Due to its central role in different biological processes, deregulated PKA activity can be found in different cancers and other diseases [23]. Somatic point-mutations for example in the catalytic subunit of PKA, are leading to Cushing's syndrome formation [24].

PKA activity also plays an important role in cell cycle regulation, because multiple signaling players are directly affected by PKA via phosphorylation such as proteins involved in anaphase-metaphase transition [25].

On the other hand, PKA can interact with protein members of other important cellular signaling pathways such as the Sonic hedgehog or the RAS-RAF-ERK pathway [26, 27]. The process of interlinking pathways is currently considered as a very important step for the development of new strategies for therapeutic interventions and to circumvent undesired off-target effects.

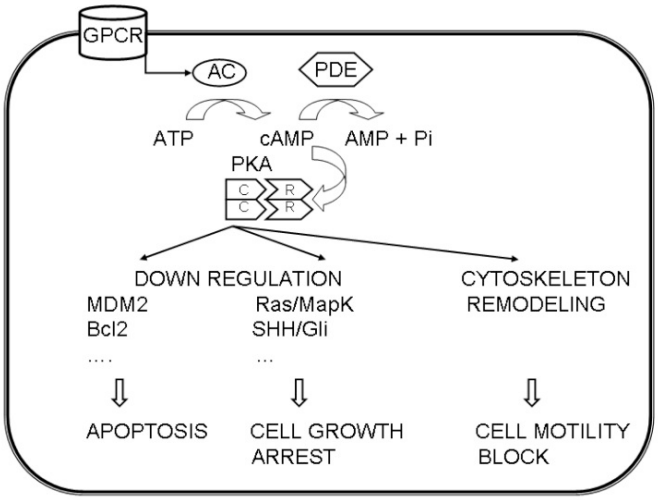

Figure 2| Scheme of the possible effects of PKA activation in cancer: Activated adenylyl cyclase (AC) leads to the production of high amounts of cAMP. Upon binding of cAMP to the regulatory (R) subunits of PKA, the catalytic subunit (C) is released and can phosphorylate several target genes, leading to different cellular responses such as cell growth or apoptosis. Picture adopted from Caretta et.al. [28].

cAMP signaling regulates a huge variety of different cellular processes. Protein expression level regulation which in turn controls gene transcription via transcriptional activators, (that is, the cAMP response element binding (CREB) protein) is one of these processes. Besides that, PKA can also interfere with cell cycle by controlling protein degradation via the proteasome and autophagy process.

As mentioned before, PKA is found to be involved in disease formation such as cancer. There are different postulated reasons for this. I just want to mention three which might be relevant in the context of my project.

One is the fact, that cAMP can regulate cell cycle. It is of interest that activity of the cAMP-PKA axis can either boosts or reduces cell-type specific proliferation.

Another reason might be the expression of regulatory subunit I and regulatory subunit II. RIa-inactivating mutations (germline or somatic mutations) are responsible for higher PKA activity which is connected to aberrant PKA signaling (Bossis and Stratakis, 2004).

Another function, in which PKA might be dysregulated is the actin-based cell migration, that involves in cytoskeleton remodeling. [25]

Due to its important role in cancer, PKA selective targeting in antitumour strategies has become very attractive:

1. cAMP analogoes: these compounds are capable of binding to cAMP binding pocket of the regulatory subunit.

2. ATP competitive small molecules, binding to the ATP binding pocket and thereby hindering the binding of ATP.
3. Targeting AKAPs, and thereby trying to influence the shuttling and transport of PKA to different cell compartments [23].

Although there are so many different ideas and possibilities of targeting PKA, several obstacles including permeability into cells, susceptibility to proteases and potential immunogenicity are leading to bad clinical applicability.

Therefore, alternative strategies of interfering with aberrant PKA have to be evaluated and established.

Our system biology-based approach might give new insights in PKA signaling and might discover new and relevant data which can be used to decipher open questions concerning PKA signaling.

A very interesting link which is currently under investigation in our lab is the control of metabolic processes such as glycolysis by PKA.

2.4. Signaling in colorectal cancer

Due to its high correlation of deregulated signaling in colorectal cancer, I also want to describe general signalling in colon cancer. In recent years, colorectal carcinogenesis (CRC) has imposed a major health burden. It shows up the second highest cause of cancer deaths in women, and the third highest cause of cancer deaths in men [29]. There are a lot of different signalling pathways involved in the formation of colon and rectum cancers. By blocking these pathways or special signalling points in the pathways via drugs, cell death should be initiated, leading to apoptosis. Here, I just want to mention two different pathways which are highly relevant for formation of colon cancer.

- The first is TGF-β/SMAD singling pathway:

The first event of the signalling cascade is a ligand-induced oligomerization of the TGFBR1 serine/threonine receptor kinase. This leads to the progression of the signal which subsequently leads to phosphorylation of SMAD proteins (Smad1, Smad2 and Smad3). These proteins are capable of interacting with each other but also with other important regulatory proteins. The last event in this cascade is the interaction with SMAD4 which is then transduced into the nucleus, where it is responsible for triggering several biological responses via transcriptional activation [30].

- The second is the MAPK kinase pathway:

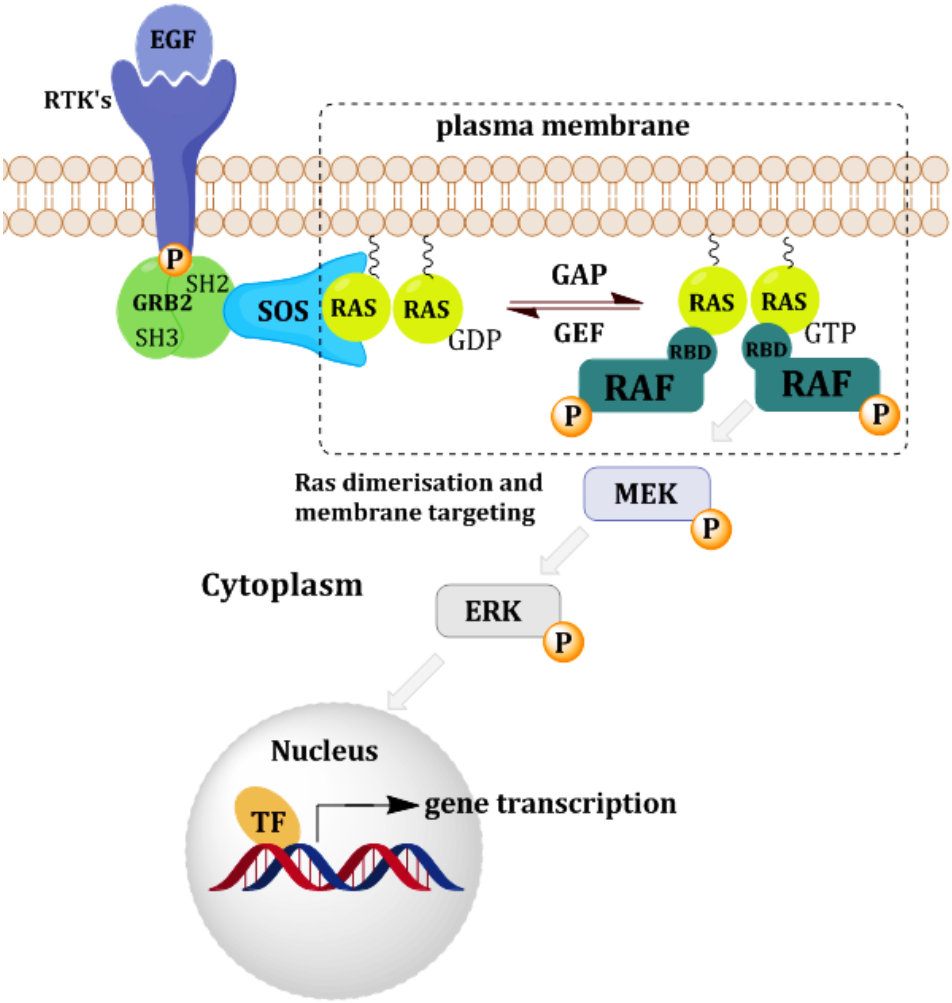

Figure 2| RAS-RAF-ERK-pathway. Upon binding of an epidermal growth factor (EGF) to the receptor-tyrosine kinase (RTK) a downstream signaling cascade is initiated. The result is a recruitment of different kinases and the mutual activation of phosphotransferase activities. Beyond this, we underline here the existence of a RAS homodimer. At the plasma membrane, the GTPase RAS binds RAF dimers and initiates several downstream phosphorylation events. The result is the phosphorylation of ERK which has a direct impact on gene transcription in the nucleus. RTK…. Receptor tyrosine kinase; EGF…. Epidermal growth factor; SOS…. Son of Sevenless; P…. Phosphorylation; GTP… Guanosine-triphosphate; GDP…. Guanosine-diphosphate; GEF…. Guanosine exchange factor; GAP…. GTPase-activating protein; TF…. Transcription factor

Upon binding of epidermal growth factors (EGFs) to receptor tyrosine kinases (RTKs), a downstream signaling, involving a cascade of phosphorylation events, is initiated [31]. The phosphorylated tyrosines of RTKs interact with docking proteins like GRB2 via the Src homology 2 domain (SH2 domain). This Src homology 2 domain represents a phosphotyrosine-binding module and is involved in changing protein localization and

regulation of kinase activity [32]. GRB2 further binds to guanosine exchange factors (GEF) like son of sevenless (SOS). The molecular SOS complex is able to activate RAS by promoting the exchange of GDP to GTP via the Src homology 3 domain (SH3) [31, 33]. Subsequently GTP-bound RAS molecules recruit RAF dimers to the plasma membrane [34]. Subsequently to RAS binding, the phosphorylated RAF dimer activates MEK by phosphorylation. In the next step MEK mediates phosphorylation of ERK. ERK activates transcription factors such as the main cancer driver MYC via phosphorylation which finally leads to the reprogramming of nuclear gene expression.

2.5. PFKFB3

Glycolysis is the energy producing pathway in our cells. Using different enzymes, glucose will be broken down into two three-carbon compounds which thereby generates energy in form of ATP [35]. One of these important enzymes is 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3 (PFKFB3). It is a critical regulator of glycolysis control, because it is responsible for the synthesis and degradation of fuctose-2,6-bisphosphate (F2,6BP) out of fructose-6-phosphate (F6P).[36] This is one of the main rate-limiting steps in glycolysis, because F2,6BP functions as allosteric activator of 6-phosphofructokinase-1 (PFK-1), which in turns stimulates glycolysis. Besides its important function in glycolysis, PFKFB3 can regulate cyclin-dependent kinases such as CDK1 or CDK4 which links this metabolic kinase to cell proliferation and survival in tumor cells [36, 37].

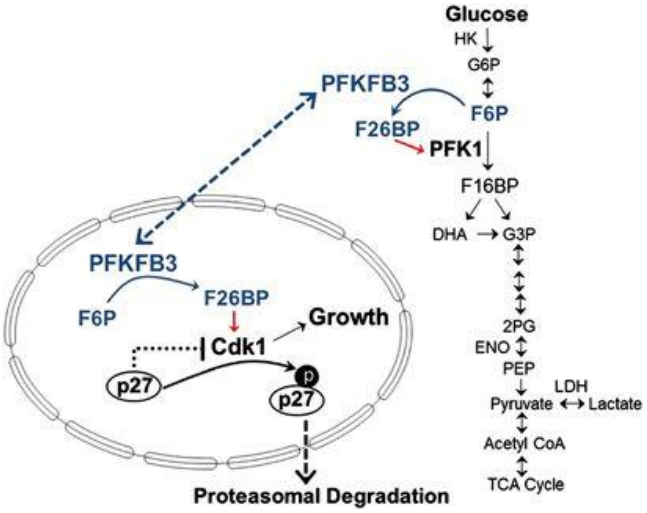

Figure 3| Schematic representation of glycolysis and PFKFB3 function in the nucleus: Glucose is converted to F6P which is subsequently transformed to F16BP by PFK1. In parallel PFKFB3 can synthesizes F2,6BP, which in turn activates PFK-1 and thereby glycolysis. PFKFB3 can be also shuttled to the nucleus where it can indirectly activate proteins involved in cell cycle control such as CDK1 and p27 protein levels. Picture adopted from Yalcin et.al. [38]

Due to the fact, that high glucose consumption is a typical hallmark of cancer cells, PFKFB3 was hypothesized to be a good target to interfere with cancer cell proliferation. Besides that, PFKFB3 is being described as a transcriptional target for oncogenic factors such as HIF-1α and is highly expressed in a lot of different cancers [39]. Right now, there are compounds available which selectively target the ATP binding pocket of the kinase. One inhibitor which is currently in clinical trial is PFK-158 [40]. First *in vitro* and *in vivo* experiments state PFK158 to be very effective in a broad range of cell lines. The treatment of this compound resulted in reduced glucose uptake, as well as induction of apoptosis in gynecologic cancer cells [41].

Moreover, PFKFB3 is a very interesting target for cancer treatment because of its role in the Warburg effect. The Warburg effect was first described 1920 by Otto Warburg. Instead of using the more efficient process of mitochondrial oxidative phosphorylation, cancer cells are switching to the consumption of lactate (anaerobic glycolysis) in order to produce ATP [42]. This process is known as the Warburg effect and is a typical feature of fast proliferating cells.

3. Previous observations in the lab

Deregulation of receptor-controlled kinase pathways decisively contributes to the formation and progression of cancer. We hypothesized that the elucidation of whole kinase interaction networks will help to explain the molecular basis for deregulated kinase activities and will help to unveil key modulators, responsible for imbalanced cancer cell proliferation. To do so we decided to use a network-based system-biology approach, which offers a good comprehensive understanding of complex biological processes. Complex diseases such as cancer cannot be treated effectively by interventions using just one single drug. However, network biology will help us to understand the mechanism of drug action and thereby improve the development of precision medicine.

In order to decipher the macromolecular architecture of deregulated PKA signaling we decided to affinity isolate endogenous PKA complexes from different colon cancer cell lines (KM12, SW620, SW480, SKCO1 and SNU-175) and Glioblastoma biopsies. We used these cell lines because they depict a special mutation at the Gα-subunit of G-proteins which results in a permanent active adenylate cyclase and therefore high amounts of cAMP and hyperactive PKA activity.

We used a cyclic AMP antagonist (Rp8-AHA-cAMPS-beads), immobilized on agarose beads to pulldown the regulatory subunit of PKA [5]. This enabled us to isolate the whole macromolecular PKA complex (regulatory and catalytic subunit, AKAPs, other interacting proteins). In a second step, we performed an on bead tryptical digest followed by LC-MS/MS analyses. After data sorting and bioinformatic data analyses (removal of redundant peptides, introducing of a uniform protein identifier, matching the phosphorylation side to each peptide, considering abundance or the protein) we were able to generate a PKA centered protein interaction network which was subsequently used to identify and select proliferation relevant substrates for further analyses.

Considering results from our Go enrichment analyses along with the process of data-matching with the Drugbank (https://www.drugbank.ca/) database we were able to select a bunch of proliferation relevant proteins for further analyses. One interesting and cancer related protein was 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 3 (PFKFB3). We decided to analyze this protein more into detail because it depicted

a prospective PKA phosphorylation site and a major player in sustaining the high energy turnover in cancer cells. This seemed to be very promising for us because it could function as a major player in the crosstalk between PKA signaling and energy metabolism such as glycolysis.

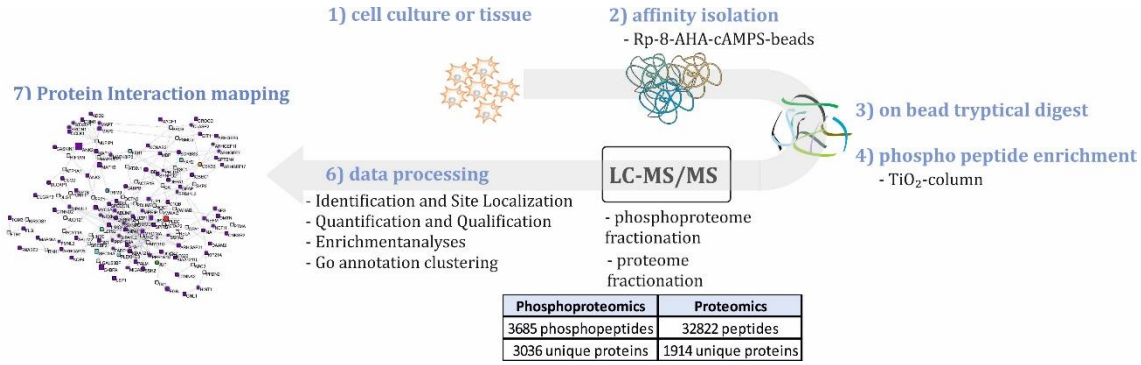

Figure 4| Flowchart for the generation of a PKA network: Affinity-isolated PKA complexes from cells or tumor tissue were analyzed using LC-MS/MS followed by semiquantitative interrelationship analyses and GO enrichment analyses in order to generate a protein-interaction network.

So far we were able to show, that PKA phosphorylates PFKFB3 at position S461 in *in vitro* phosphorylation immunoprecipitation experiments. Interestingly, this position (S461) is directly linked to activity of PFKFB3. Supringsly, we also examined a nuclear localisation of this protein which seemd to be interesting, because its major task is to function in glysolyses, a basic biochemical process whioch occurs in the cytosplasmic part of the cell. Besides that we were able to show that inhibition of PFKFB3 by PFK158 (selective PFKFB3 inhibitor) is leading to an antiproliferative effect in the different colon cancer cell lines.

4. Aims of the study abroad

Besides other ongoing investigations we decided to subdivide my research efforts in San Diego into 3 aims.

- **Further evaluation of the proteomics data set** (moth 1)

I planed to perform this analyses in collaboration with the proteomics facility at the Cancer Moores Research Center in La Jolla. I assessed one month for this evaluation including the preparation of the final figures. The basic plan was to further process the proteomics data set and the phospho-proteomic data set in order to generate some usefull figures for a better and easier illustration of the generated data.

- **RNA seq correlation** (month1)

I planed to perform the correlation of RNA sequencing data with my generated proteomics data in cooperation with the bioinformatic facility at the Cancer Moores Research Center in La Jolla. I also assessed one month for this evaluation including the preparation of the final figures. The generall aim was to correlate our data with RNA sequencing data from the Gutkind lab and thereby monitor gene expression and transcriptome changes within the different cancer cell lines. Such an approach can help us to determine other relevant proteins, expressed in the different cancer samples. The idea was to introduce coloured bars next to the nodes which illustrate the fold change (p-value) relative to a control (GNAS wild type cell lines such as SW480 or SW620). Moreover we planned to highlight protein hits from the network which show a significant fold change of RNA expression and a significant enrichment (illustrated by the size of the spots) in the protein-interaction-network

- **Performing of a RNA-Tuc-Seq labelling and subsequent RNA sequencing analyses in colon cancer cell lines** (month 2 and 3)

Based on a publication of Riml et.al. we planned to perform mRNA labeling of colon cancer cell lines treated with our PFKFB3 inhibitor and subsequent RNA seuquencing and bioinformatic analyses. We assessed two months for this because of the intense bioinformactic data analyses process. We planned to look at the reprogramming of gene expression as a result of PFKFB3 inhibition or activation (using a mutated and active form of the protein). To do so we initiated a metabolic RNA labelling [43] which

should enable us to study the dynamics of newly transcribed mRNAs upon kinase inhibition. We decided to apply a novel sequencing method called TuC-sequencing (Riml et.al. 2017) that eliminates affinity purification and allows for direct assessment of 4sU-labeled RNA to identify direct gene targets.

- **Patient derived xenograft analyses** (month 1, 2, 3)

The Gutkind lab has a high expertise in *in vivo* exeriments with mice. We planned to perform xenograft studies of mice, treated with the selective PFKFB3 inhibitor. We assessed around three months for this study. Besides that, I planned to attend different trainings, dealing with rodent handling such as injections or oral gavage.

5. Results

5.1. Further evaluation of the proteomics data

We set out to further analyse our proteomics data set in order to identify prospective interaction partners of PKA. With the normalized signals of the peak area from our MS/MS experiments we were able to calculate the fold change between the proteomics sample and the cAMP controle. This gave us a more comprehensive understanding of the generated data. By using this approach we saw, that our startegy was working quite well. The blue dots indicate a high fluctuation in the proteomics and the cAMP control, wherease the red ones should indicate prospective interactors of PKA. We were able to identify the two reguatory subunits of PKA (PRKAR1A and PRKAR2A) at high fold change and high log p value which indicates that they were highly enriched in the proteomics data set but not in the cAMP control. This is in perfect accordance to our hypothesis, because the cAMP should interrupt the binding of the regulatory subunits to the cAMPS- beads. Interestingly we were also able to see enriched prospective binders like Oxoglutarate Dehydrogenase (OGDH) or Lactate Dehydrogenase B (LDHB), proteins involved in metabolic processes.

Nevertheless we have to concider that still unspecific binding to the beads can occur. Especially nucleotide binders (e.g. GMP binding proteins or ATP binding kinases) tend to bind to these beads. Therefore we also annotated all the nucelotiode binder in our network.

We now plan to analyses some of the identified proteins for *in vitro* interaction studies such as GST-pulldown or immunoprecipitation experiments. Due to the high number of intersting proteins we decided to use a method called Lumier assay in order to profile the interaction pattern. Following overexpression of NanoLuc-tagged prey proteins (prospective interaction partners such as OGDH or LDHB) and PKA variants (bait) we pulldowned the bait protein and detected the emitted light of co-purified prey proteins after incubation with benzyl-coelenetarzin. This should help us to characterize the modes of PPI. Because LUMIER is highly scalable (96 well-plates) it is a valuable tool for us to identify and characterize dynamically regulated PPIs in mammalian systems.

So far we were able to show interaction of the catalytic subunit of PKA with PFKFB3, PFKFB1 and TRIM33. Nevertheless we also observed, that all these proteins are only interacting with the catalytic subunit but not with the regulatory subunits of PKA. The

results of the Lumier assay with the different other interesting proteins form the proteomics are still subject for ongoing experiments.

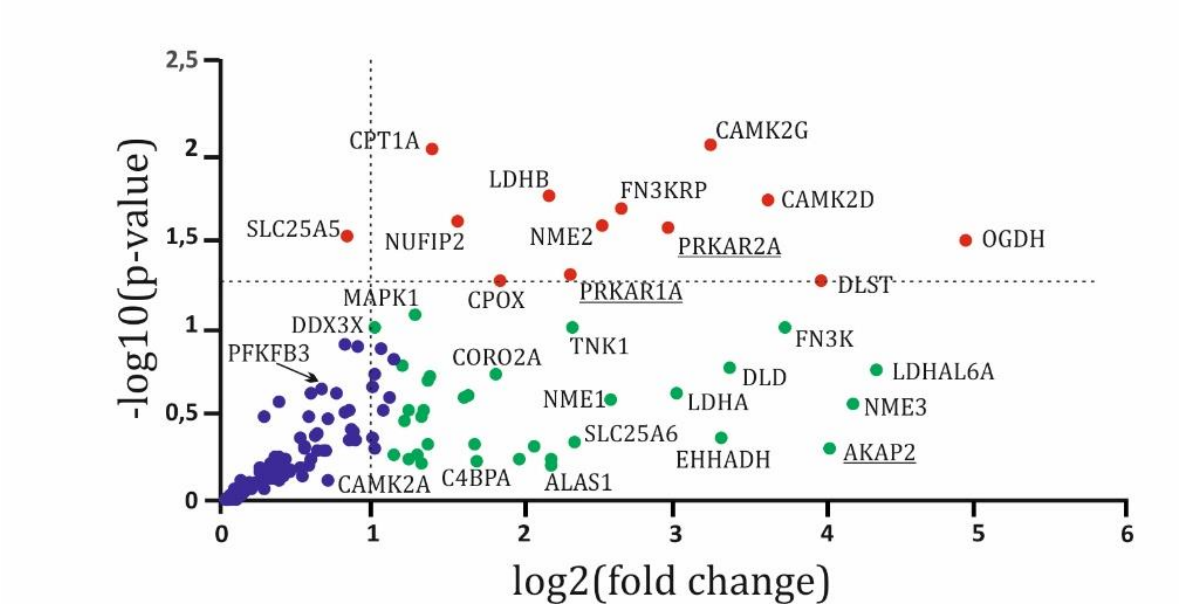

Figure 5| Determination of prospective interaction partners: Logarithmic ratios of proteomics versus cAMP control experiments are plotted against the negative logarithmic p-value. Proteins located in the blue area are considered putative unspecific interaction partners whereas proteins in the red area are prospective interactors of PKA.

In a next step we started to functionally annotate the data set using Gene Ontology terms. To do so we used an online enrichment toll (www.cpdb.molgen.mpg.de). Thereby, genes are clustered into functional terms such as “molecular function”, “cellular component” or “pathway overrepresentation”. As a readout we used the log10 of the p-value which is calculated based on the overrepresentation of the protein in a special Go term. As background we used the whole dataset we gained from our MS/MS analyses. The enrichment analyses indicated a high enrichment of proteins involved in PKA activation and G protein signaling which was again in perfect accordance to our hypothesis. Indeed we were able to enrich the different subunits of PKA alsong with whole bunch of A kinase anchoring proteins (AKAP’s). Besides that we saw an enrichment of proteins involved in Insulin signaling and thereby in energ metabolism processes.

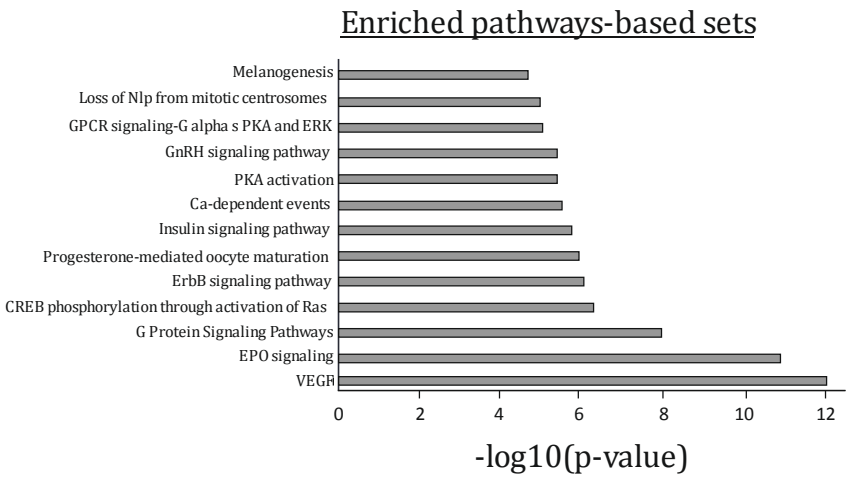

Figure 6| Pathway enrichment analyses: pathway enrichment analysis was performed using www.cpdb.molgen.mpg.de. The enriched pathways are presented as -log10(p-value).

5.2. RNA sequencing correlation

The basic idea of correlating our proteomics data with RNA sequencing data form the Lab of S. Gutkind was to narrow down some genes which might be affected by PKA. Our strategy was to overexpress a doxycycline (Dox) inducible mutated GαS-protein, characterized by the GNAS activating mutation (R201C) which should activate the adenylate cylce and PKA. As a negative controle we perfomed the same experiment in the absence of doxycycline. In a first step we overexpressed the Dox-inducible GNAS plasmid in DLD1 colon cancer cell lines. This cell line is GNAS wilde type and shows normal cAMP production. Second, we exposed the cells to doxycyclin for 12 h, harvested the cells and isolated the RNA using a comerical available kit from Quiagen. The isolated RNA was send for deep sequencing and subsequently to the bioinformatic facility. After 3 weeks we got the results and started to correlate this data with our processed proteomic database in order to create a final figure. To do so we used the fold change scores (values, indicating how much a quantity changes going from an initial to a final value) of the RNA sequencing above a special treshold (which is illustrated in figure 7 by horizontal white lines) along with the criteria of a maximum found peptide score (number of unique identified peptides) of 2 from our proteomics dataset. Another criteria was to use only proteins in our network, which are said to be druggable (based on www.drugbank.ca) by a special compound or an approved drug.

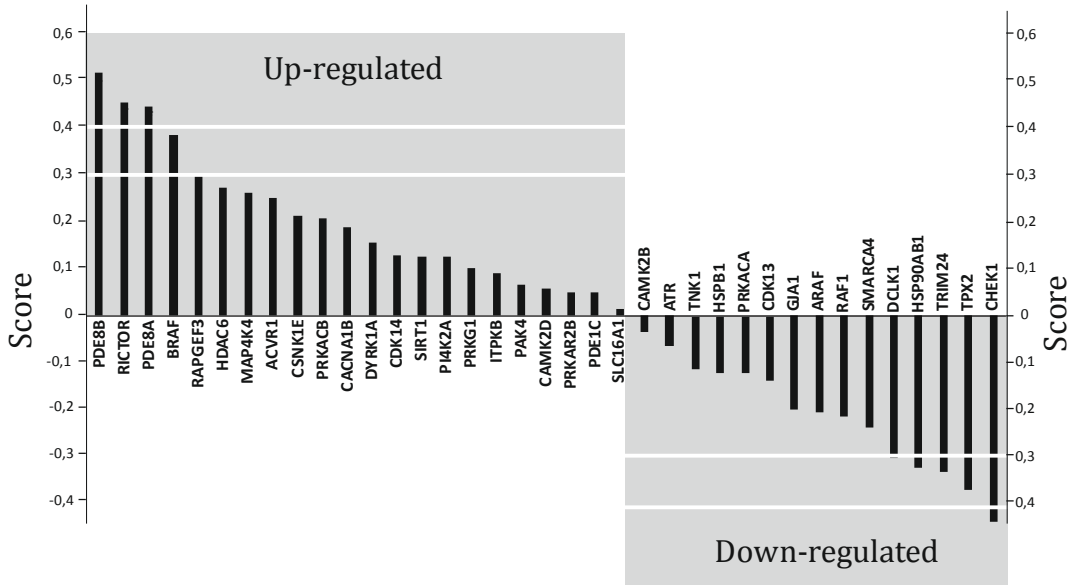

Figure 7| Correlated RNA-seq fold change values: Bar plot indicating the fold change values obtained for 37 differentially expressed genes in DLD1 colon cancer cell line which are druggable candidates of the PKA interaction network.

This overview can now be used to select proteins from the network in a more systematic way in order to analyse its impact on PKA driven cancer cell proliferation. We identified proteins involved in pathways linked to cancerogenesis such as mTOR or MAPK signaling. But besides that we were able to detect proteins linked to cell cycle such as CHEK1 kinase. In a next step we want to look for prospective PKA phosphorylation sites which might regulate the functional outcome of these proteins. This might be a good starting point to initiate new projects. In preliminary experiments we were able to show a cAMP dependant upregulation of endogenous RICTOR protein level after expousre to the PKA stimulator Forskolin. We also tested prospective phosphorylation of RICTOR by PKA but were not able to show phosphorlylation by this kinase.

5.3. RNA-Tuc-Seq labelling and RNA sequencing analyses in colon cancer cell lines

PFKFB3 is generating fructose-2,6-bisphosphate which in turn stimulates glycolyses. Besides PFKFB3, there are three other members (PFKFB-1,2 and 4) of the PFKFB family, involved in gylcolyses and energy metablism. Interestingly only PFKFB3 is uniquely localized in the nucleus [44]. The reason for that is still not completely understood. PFKFB3 is said to partly shift the glycolyses into the nucleus. Another reason might be the interaction and regulation of proteins involved in cell cylce controle such as CDK4 or CDK1 [7, 45].

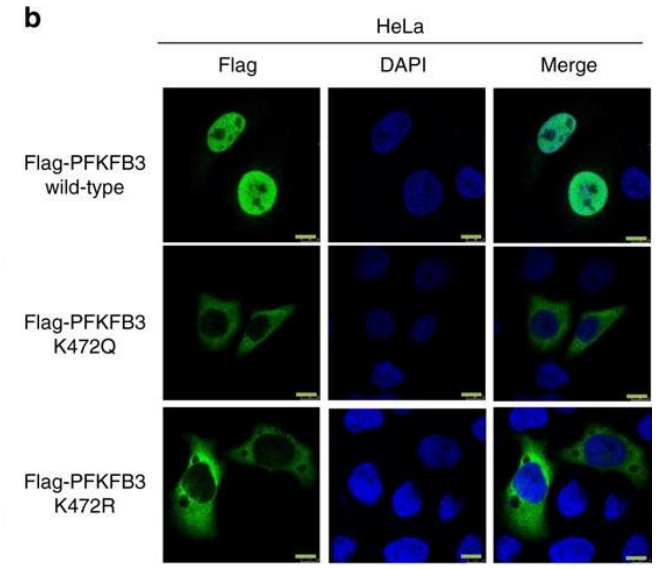

Figure 8| Localization studies of PFKFB3 wt and K472 mutant: Indicated PFKFB3 plasmids (WT, K472Q or K472R mutant) were transfected into HeLa cells and immuno-stained with anti-Flag antibody and with a nuclear marker. Picture adopted from Li et.al [44]

To understand the functional role of PFKFB3 in the nucleus we planned to elucidate the dynamics of gene expression upon PFKFB3 inhibition with the selective kinase inhibitor PFK-158 (from Sellechem). We decided to use a new established method from Riml et. al. [43] in oder to label newly transcriped mRNA with the metabolic labeling reagent 4s-thiouridine (4sU). First we treated our colon cells with 4sU for 1 h in order to incorporate enough 4sU into the cell. Afterwards we treated our cells with 1µM PFK158 for 1h followed by harvesting the cells and extracting total RNA using a precipitation method [46]. The last step included the treatment of the isolated RNA with OsO_4 at a concentration of 1mM to transform 4sU into cytosine. This reaction was carried out at room temperature for 1h and in the presence of a NH_4Cl buffer, which

was freshly prepared pefore carrying out the reaction (see methods). The isolated total RNA was then shipped to Microsynth® for mRNA enrichment and illumina RNA sequencing. The obained raw data set was then analyzed by the bioinformatic facility at the Cancer Moores Research Center in La Jolla.

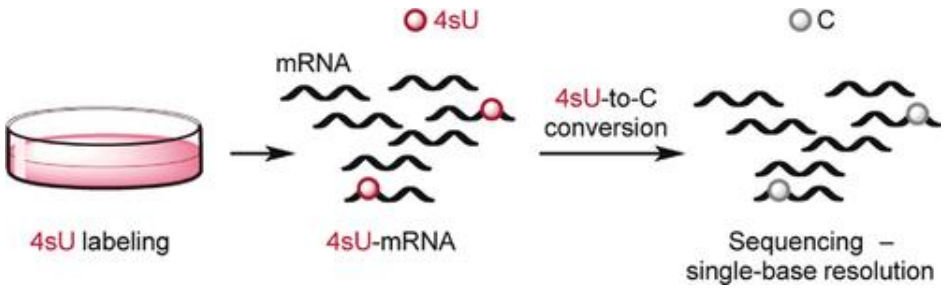

Figure 9| Process of metabolic labeling using 4sU: After treatment with 4sU, total RNA was isolated from colon cancer cell lines. The labeled and newly transcribed RNA (4sU RNA) is then chemically converted to C using OsO_4. Picture adopted from Riml et.al. [43].

This is an still ongoing process because of low vacancies of the bioinformatic facility and the difficulties in data acquisition. Nevertheless we were able to map our PFKFB3 gene into a previously established CRISPR/Casp viability screen in order to score for a few relevant genes vs. different phenotypes (Kras, etc). The profiles of beta-catenin, KRAS and HNF4A were used as a positive control as they are top lethalities of the PKA state. We were able to detect a modest decrease of cell viability with the silencing of PFKFB2 but quite less for PFKFB3 or PKFM. We still have to evalute this more into detail.

Besides the RNA Tuc Seq analyses we are also interested in performing normal RNA sequencing analyses and immunostaining of PFKFB3 in colorectal cancer cell lines.

5.4. Xenograft analyses in colon cancer cell lines

In the process of drug development, the majority of compounds going into clinical trial fail because of either lacking efficacy or saftey [47]. By the use of patient derived xenografts (PDX) in the preclinical phase, the pharmaceutical companies try to increase the prediction of efficacy of certain compound. For PDX studies, patient derived cancer cells are directly implanted into immunodeficient mice. After growing of the tumor, drugs and other small molecules can be administred to the mice and the size of the tumor can be determined as a fuction of drug dosage.

Our initial proliferation assays in different colon cancer cell lines depicted a significant antiproliferative effect of PFK158 kinase inhibitor, indicated by a dose dependant downregulation of cell viablity. We then decided to test the impact of this drug *in vivo* during my stay in San Diego. To do so we initiated patient-derived Xenograft studies at the Moores Cancer Center in La Jolla. We injected 2 mio. cultured cells of the KM12 colon cancer cell line and 2.5 mio. cells of the SW620 colon cancer cell line into nude and immuncompromised mice. After reaching a tumor burdon of around 150-200 mm^3 we startet to inject 25 mg/kg of PFK158 intraperetoneal (i.p.) every thre days. Due to the low solubility of the compound, we used a special vehicle for injection, consisting of Tween80, PEG and PBS. We prepared a stock solution of the compound of 100 mM and diluted it down to 10 mM with the vehicle. As a controle we administred the vehicle (DMSO, diluted in the special vehicle to 10 %) alone. Still the compound was not completely soluble but we managed to get a good suspension which was feasible for injection.

Unfortunately we were not able to see a reduction of tumor size after 5 injections. Due too high tumor burdon and because of animal welfare we had to euthanize the mice. We went back into the literature and decided to start a new trial in two weeks. This time we planned to admister the drug in DMSO and to start the treatment at a tumor size of about 90 mm^3 to max. 100 mm^3. Due to the fact, that PFK158 is a metabolic inhibitor, it might be possible that it works primarily in small tumors and needs to be enriched in the tumor tissue. We decided to dilute our compound to about 20 mM in DMSO so that the final admistred DMSO/Compound volume does not exceed 100 µL. To monitor the wellfare and healthiness of the animals we also decided to measure their body weight every three days after injection. This should give us a good indication of toxicity of the

compound or DMSO alone. Unfortunatly the mouse facility was contaminated with a skin bacteria which also infected our mice. Due to animal welfare we had to euthanize the mice in a very early stage of the treatment process. Neverthelss we were able to see a small reduction of tumor size after 2-3 injections.

As a conclusion we were not able to show a significant decrease in tumor volume. Nevertheless we were able to establis a good and robust protocoll for injection and treatment procesdure for future studies. We now plan to repeat these exeriments in the presence of other drugs, looking for synergistic effects. As a starting point for the xenograft analyses we want to use the extracted information from the RNA Tuc-sequencing. In the case of interesting up or downregulated mRNA's we would like to initiate proliferation assays *in vitro.*

6. Methods

- **Patient derived xenografts (PDX):**

- Cultivate colon cancer cells until they reach high cell confluency.
- Add Trypsin/EDTA (1x) to the cells and incubate them until they are all unattached to the dish.
- Harvest the cells by centrifugation at around 1000r rpm for 1 min at room temperature (RT)
- Resuspend the cells twice in PBS and centrifuge again at 1000 rpm for 1 min at RT.
- Remove PBS and resuspend the cells in the appropriate media without FBS or antibiotics.
- Count the cells and remove an aliquote for the injection. Keep tube on ice.
- Inject the appropriate amount (around 2 million cells per flank) subcutaneously in the two flanks of the mouse.
- Measure the tumor size every three days until the tumor reaches around 150-200 mm^3
- After reaching the desired tumor size start application of the drug.
- Measure the tumor size every three days during the first two weeks and afterwards every day.

- **SDS page**

- Assemble two clean glass plates separated by a spacer.
- Prepare the appropriate volume of *Separation gel solution* containing the desired acrylamide concentration (e.g. 10 %, 12 %, or 12.5 %).
- Add APS and TEMED and mix the solution and pour it into the gap between the glass plates.
- Carefully overlay the Separating gel with isopropanol.

- After complete polymerization pour off the overlay and wash the top of the gel with deionized water.

- Prepare the appropriate volume of *Stacking gel solution,* add APS and TEMED and pour it directly onto the surface of the polymerized separating gel. Put on the comb immediately and wait for polymerisation.

- After polymerization (>1 h) carefully remove the comb and rinse the slots with deionized water.

- Mount the gel in the electrophoresis apparatus and fill top and bottom reservoirs with *1x Tris-glycine electrophoresis buffer*.

- In parallel heat the protein samples to 95 °C for 10 min in *1x SDS gel-loading buffer* to denature the proteins.

- Load the samples and a size marker into the bottom of the wells using a HAMILTON microliter syringe. After each probe wash the syringe with *1x Tris-glycine electrophoresis buffer.*

- Attach the electrophoresis apparatus to an electric power supply and apply a constant voltage of 120 V for 10 min. After the dye has moved into the separating gel increase the voltage to 160 V and run the gel for 1h.

- Remove the glass plates, separate the plates and cut of the stacking gel with a scalpel.

- In case of a following Semidry Transfer the gel should be washed for about 15 min in *1x Tris-glycine-semidry-transfer buffer*.

- If no Blotting is followed, the gel can be stained with *Coomassie Brilliant Blue.*

  - **SemiDry-Western Blot and detection with ECL system**

- Equilibrate a PVDF membrane in 100 % methanol for 30 seconds, followed by 3 minutes in water and at least 15 min. in *1x Immunoblot transfer buffer.*

- Layer three pieces of Whatman papers (equilibrated in *1x Immunoblot transfer buffer)* onto electrode (anode), followed by the PVDF membrane. Put the gel on top of the PVDF, followed by three more Whatman papers. Remove any bubbles between the layers and close the device with the top electrode (cathode). Set up 9 V for about 2h.

- After the transfer stain the membrane for 2 min in cold *Ponceau S staining solution*. Destain the membrane in water and label the protein size marker. Destain the membrane with ethanol for 1 min, followed by 1× *TBS* for 30 min.

  - **Detection with enhanced chemilumineszenz (ECL)**

- Incubate the membrane in a 5 % *Blocking solution* for about 30 min.

- Afterwards wash the filter 3 times for 5 min with *1x TBS-T*.

- Apply the first antibody (diluted in blocking solution or BSA based on the manufactures recommendations*)* over night at 4 °C. Wash the filter 4 × 10 min with *1x TBS-T*.

- Apply the second antibody (e.g. anti-rabbit/mouse, coupled to a horseradish peroxidase) diluted to 1:2500 or 1:5000 in 5 ml of *Blocking solution* and incubate at RT for minimum 1h.

- Wash the filter 4 × 10 min with *1x TBS-T*.

- Mix equal volumes of *ECL-detection solution 1* and *ECL-detection solution 2* (500 µl per blot).

- Add the detection reagent on the membrane.

- Incubate for 1 min at room temperature. After 5 min drain off the excess liquid.

- Visualize the proteins using Fusion Fx7 (peqlab) imager.

- **Passaging of cells**

- Aspirate all the DMEM-medium of a 100 mm-Petri dish and wash the cells twice with 5 ml *1x TS buffer*.

- Add 1 ml of *1x trypsin-EDTA* to the attached cells and distribute equally on the dish, put the dish for 5 minutes in the incubator until the cells are released from the dish.

- Resuspend the cells in 5 ml of DMEM-medium and transfer the cell suspension to a 15 ml-Falcon-tube.

- Pellet the cells by centrifugation for 2 minutes at 1.000 rpm (Heraeus Biofuge 13) and resuspend the obtained pellet in fresh DMEM-medium.

- Dilute the cells 1:5 and distribute the cells to new 100 mm-Petri dishes containing each 10 ml DMEM, supplemented with foetal bovine serum and penicillin-streptomycin.

- **Preparation of cell-lysates**

- Aspirate the DMEM-medium and wash the cells with *1x TBS*.

- Aspirate washing solution.

- Resuspend the cells in *1x TBS* and scrape them down from the dish.

- Transfer the cells into new tubes and overlay them with 1 ml of *1x TBS*.

- Centrifuge the tubes and remove the medium.

- Dissolve the pellet in the appropriate amount of *1x SDS loading buffer*.

- Suspend the pellet in the buffer and heat the samples up top 95°C for 5-10 min.

- **TuC-Seq labelling**

- KM12 and SK-CO-1 colon cancer cellsls were seeded into 10cm dish (2 million cells in total) and grown overnight at 37°C and 5% CO2 in the appropriate media.

- On the next day the medium was replaced with new media supplemented with 0.1 mM 4-thiouridine (4sU; Jena Bioscience) and incubated for 1 hour.

- Afterwards the cells were harvested.

- The next step was to isolate the total RNA using a precipitation method [46].

- 4sU-to-C conversion reaction (see Riml et.al.)

- The OsO4 solution (1 mm) was freshly prepared from aqueous OsO4 stock solution (1 mL; 100 mm) stored at −20 °C. NH4Cl solution (2 m) was prepared by dissolving NH4Cl (10.7 g) in H2O (100 mL) and adjusting pH to 8.88 by the addition of ammonium hydroxide solution (2 m).

- 1nmol RNA (dissolved in H2O (10 µL)), NH4Cl solution (2 µL; 2 m, pH 8.88) and OsO4 solution (10 µL; 1 mm) were added to give final concentrations of 0.45 mm OsO4 and 180 mm NH4Cl in a total volume of 22 µL. The reactions were mixed and incubated for 4 hours at room temperature.

- After the incubation time the reaction was stopped by putting the tubes into dry ice.

- The tubes were then shipped to Microsynth AG for mRNA enrichment and deep sequencing.

  - **RNA precipitation: (manual from Harvard)** **(**https://projects.iq.harvard.edu/files/hlalab/files/ethanol-precipitation-of-rna_hla.pdf)

- Add: 0.1 vols 3M Sodium acetate and 2.5-3 vol of ice cold 100% Ethanol

- Vortex to mix thoroughly. 2.

- Precipitate at -20 0°C for 1 hour or overnight or -80 0 C 1 hr (overnight will give more precipitation if RNA amount is low)

- Centrifuge at full speed (13000rpm) at 4 °C for 30 min
- Wash pellet twice with 0.5ml ice cold 75% Ethanol, spinning at 4° C for 10 mins each time.
- Take Ethanol out, spin quickly (10s top speed) to remove the trace amount of Ethanol as you can.
- Allow to Air dry the pellet and resuspend in an appropriate volume of Nuclease free water.
- Precipitating small amounts of RNA (Glycogen 20ng per sample may be added to the RNA before precipitation to aid visualization when precipitating small amounts of RNA)
- Add 1ul of a 20mg/ml solution of Glycogen (RNase DNase free)

7. Discussion

In the course of my project we set out to get a more detailed understanding of the molecular basis of deregulated PKA signaling. We therefore used a system-biology-based approach which led to the identification of key modulators, responsible for imbalanced cancer cell proliferation and therefore to the identification of proliferation-relevant substrates and druggable effector proteins for therapeutic intervention. Due to the fact, that colorectal cancer is the third leading cause of cancer related deaths in the United States [48] this topic is and was of high relevance.

One major hit from our network analyses was the 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 3 (PFKFB3), which plays a key role in glycolysis and in coordinating cell cycle [7] and was research subject for the my three months stay in San Diego, California.

During this three months stay in San Diego at the Cancer Moores Research Center in La Jolla, I was able to adobt basic skills in rodent handling and the performance of patient derived xenografts (PDX). We used this method for further analysing the effect of the selective PFKFB3 inhibitor PFK-158 (which is currently under clinical trial) *in vivo*. We managed to establish a basic protocoll for the injection of the colorectal cancer cell lines into nude mice. Further we determined the perfect concentration, formulation and time point of the drug administration into the nude mice. Nevertheless we were not able to generate final and useful data and therefore have to repeat the experiments again.

Besides the *in vivo* experiments we managed to further analyse the effect of the PFKFB3 inhibitor in *in vitro* experiments. We were interested in the effect of this metabolic inhibitor on important cell cycle proteins such as cycline E1, cyclin D1 and the phopshorylation status of the retinoblastom-protein (pRb, Rb), which is a protein responsible for slowing down cell growth by inhibiting cell cycle controlling proteins. After exposure of the compound to the colon cancer cells we were able to detect a downregulation of p-RB at high PFK158 concentration as well as downregulation of cyclin D1. This indicates an important role of PFKFB3 in controling cell cycle which should be analysed more into detail. Therefore we plan to repeat these experiments with siRNAs for PFKFB3 knockdown. We are also interested in analysing the link between CDK4 and PFKFB3. The combination of CDK4/6 inhibitors along with PFKFB3 inhibitors might be a good strategy to interfere with colon cancer cell proliferation.

Therefore we are currently performing proliferation assays in colon cancer cells, look for synergistic effects of PFK158 along with Palbocilip or Abemaclip (both selective CDK4/6 inhibitors). This might be of high interest because previous published results indicate that inhibition of CDK4/6 alone is not very sufficient to cause significant reduction in tumor burden [49]. By the combination of these drug groups we might be able to establish an alternative approach of interfering with colon cancer progression.

Another part of my research abroad was the completion of my proteomics and phospho-proteomics data set. During the three months I was able to finsih the analyses of my proteomics data and to generate a scatter blot indicating the interesting interaction partners of PKA. These hits are now subject to ongoing biochemical experiments to prove physical interaction of these proteins with Protein Kinase A.

Nevertheless there is still the open question about the role and function of PFKFB3 in the nucleus. In preliminary experiments I was able to show a link between PFKFB3 and CDK4/6. Moreover recently published data [7] underpins this hypothesis. To further evaluate and analyse the impact of PFKFB3 activation or inactivation on pathological downstream targets (e.g. phosphorylation) and nuclear signaling we initiated RNA TuC-Seq analyses. The results of the TuC sequencing are still pending but should function as a starting point for the selection of possible PFKFB3/PKA downstream targets.

Besides interesting hits from cell cycle regulator proteins we might be also very interested in SMAD proteins involved in the TGFbeta pathway. In preliminary experiments during my stay abroad I was able to detect a prospective crosstalk between PKA and TGFbeta signaling in which PFKFB3 might play a central role. By overexpressing PFKFB3 in colon cancer cells I was able to detect an upregulation of phospo-SMAD2 whereas the endogenous SMAD2 and SMAD 3 protein levels seemed to be unaffected by the overexpression. We speculate that the combinations of PFKFB3 modulators with other cancer drugs (such as TGFbeta receptor antagonists) may have the potential to increase the efficacy of specific cancer drugs. We are aware that this is quite ambitious.

In the likely event of a promising proliferation assays showing synergistc effects and interesting hits from the RNA seq analyses we further plan to analyse the impact of combination therapy on tumour growth in patient derived xenografts (PDX). We plan to inject colon cancer cells into mice and expose the animals to preselected drugs. Due

to my recent stay at the Cancer Center in La Jolla and due to the high expertise in the field of *in vivo* drug screens, we contemplate to cooperate with Silvio Gutkind at UCSD in California.

8. Resume

Taken all together, the support of the Marshall Plan Scholarship helped me to further advance my PhD project. I was able to further identify proliferation relevant proteins form my PKA centred protein interaction network which are involved in colon cancer proliferation and somehow linked to PKA activity. By the use of this information I lay the foundation for further projects in our lab.

By the help of Prof. Gutkind we also encouraged our project in such a way, that we identified novel mechanisms in which deregulated PKA activity or GPCR's contribute to the progression of colorectal cancer. Furthermore we strengthened the cooperation between our lab and the research group of S. Gutkind.

I learned a collection of cutting-edge technologies for studying cancer etiology and progression at the molecular level and in the living subject (mice).

Besides that I had the unique opportunity to join an international highly ranked laboratory. I gained basic insights into pharmaceutical cancer research and was able to advance my basic knowledge about cancer signalling and *in vivo* mouse work.

Furthermore I had the chance to advance and expand my biochemical knowledge, my laboratory and my English language skills.

As a scholar of the Marshall Plan Association in the year 2019 I was able to expand my horizons, getting to know fascinating people and educating myself on a broad basis. This was a great chance for me and I am very thankful for the financial support.

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