# Effect of modulating IRS-1 PH domain interacting protein expression in the progression of lung cancer

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# I. Abstract

Cancer is gaining more and more importance as the number of cases rises each year. Although modern medicine has made a lot of progress and we gained knowledge that is helping us to treat patients better there are still many cancers that don't respond well to classical treatment. The reason for this is the unlimited variation of mutations within the tumor, making it nearly unpredictable. Gaining knowledge about the proteins involved in cancer progression is helping physicians and scientists to develop new strategies to fight cancer.

Lung cancer is the most prominent cancer, especially in men and accounts for every fourth death related to cancer worldwide in both, men and women. Also lung cancer is one of the hardest to detect and cure, leading to a low survival rate of lung cancer patients.

To find suitable markers and also targets in difficult to treat tumors we were focusing on novel pleckstrin-homology domain interacting protein (PHIP) and its role in tumor progression. It was detected that PHIP levels are especially high in metastasized melanomas and PHIP was therefore suggested as a prognostic marker for the metastatic potential of cells as well as for ulceration and therefore the overall progression of the disease.[1,2]

In this project we wanted to explore the role of PHIP in other cancers that were also found to overexpress PHIP. We downregulated PHIIP expression in a lung cancer cell line and after confirmation of protein knockdown, assays were performed to assess the effects of modulating PHIP expression on proliferation and invasive ability of targeted cells as well as tumor growth *in vivo* of. We conluded that PHIP is significantly enhancing the proliferation and therefore growth of the cells as well as the invasive ability which is a driving factor for metastasis in vivo. This potential of the cells to drive tumor progression was also confirmed *in vivo*.

We suggest PHIP as a new rational target for the treatment of lung cancer patients.

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# 1. Introduction

## 1.1. Lung Cancer in General

Cancer is the second most frequent cause of death in the world and the numbers of new cancer cases is not declining. With around 14 million new cancer cases worldwide and approximately 8 million deaths in 2012, lung cancer is the most frequent cancer in men, with 16.7 % of all cases in 2012 and an incidence rate of 34.2 cases per 100,000 men.

For 2016 there were over 1,6 million projected new cases in the US alone, regardless of the type of cancer, and nearly 600 000 deaths.

For lung cancer especifically there were around 225,000 estimated new cases in 2016, around 10 000 more men than women will be affected. 160,000 people were thought to die of lung cancer in 2016 and here there are again around 10,000 more men than women affected. This means that lung cancer accounts for nearly one fourth of all cancer related deaths.

When looking at the estimated lung cancer incidences in the whole world in 2012, one can see that also here 16.7 % of new cancer incidences in men were lung cancer, compared to only 8.7% in women.

To conclude, it can be said that lung cancer is one of the most popular and hard to cure diseases. The fact that men are at much higher risk to get lung cancer and die from it can be related to the smoking habits of men and women, as smoking is directly related to lung cancer. [3,4]

But of course smoking is not the only cause of lung cancer. As any other cancer, also lung cells are prone to mutations. In general lung cancer is divided into two large groups. One is the non-small cell lung cancer (NSCLC) and the other small cell lung cancer (SCLC). With approximately 80-85% NSCLC is the most common type of lung cancer and the remaining 10-15 % are considered to be SCLC. It is important to discriminate between those two lung cancer types as the decision for a treatment varies greatly.

NSCLC has a lot of subtypes as to the different cell types affected. Adenocarcinoma is the most common lung cancer type and occurs mainly in smokers

but also in non-smokers.
Squamous cell carcinoma, also called epidermoid carcinoma is the second most frequent type of NSCLC and can be found in young squamous cells that line the interior side of airways and have a flat shape.
Large cell carcinoma, also a subtype of NSCLC, describes undifferentiated cells that originate from epithelial cells in the lung and accounts for around 15% of NSCLC. There are also other subtypes but they are much less common as the ones named above and would include sarcomatoid or adenosquamous carcinoma.[5]

Since 1980 it is generally known that smoking has a direct correlation to the risk of getting lung cancer and that quitting smoking decreases this risk, so the less years a person smoked, the lower the risk of getting lung cancer, independent on the specific subtypes.
Table 1 shows results from a study conducted from 1976 to 2002, which were getting used and revised in another study in 2008. It shows the relative risk to get one of the lung cancer subtypes for people that smoked for a specific period of time and were currently smoking when the study was conducted, so for people that did not quit smoking.
The relative risk is the comparison between the probability for a group of people to get a disease and the probability to get the disease in another group of people. Thereby the probability (to get the disease) for the group that is exposed to a risk factor is divided by the probability for the group that is not exposed to the risk factor.
Here the risk factor is smoking, and the years of smoking define the group of people. The results show that small cell carcinoma is definitely the most sensitive to smoking, with a relative risk of nearly 80 and 110 cases after 40+ years of smoking, compared to 2 cases in non-smokers. Large cell carcinoma seems to be most resistive to smoking with a relative risk of 3.57 and 23 cases in 40+ years of smoking compared to 11 cases in non-smokers.
Also remarkably is the number of patients having adenocarcinoma although they have never smoked. With 85 cases in adenocarcinoma and 11 cases in large cell carcinoma these two subtypes seem to show the least effect in smoking and non-smoking,

although of course also here smoking is increasing the risk, but they have the highest rate compared to the other types in non-smokers. [6]

**Table 1**: Relative risk (RR) to get one of the subtypes of NSCLC for people smoking for a defined period of time and did not yet quit smoking. Most noticeable numbers are underlined in grey

| | Smoking Duration (years) | | | | |
|---|---|---|---|---|---|
| | Never-smoker | 1 to <20 | 20 to <30 | 30 to <40 | 40+ |
| Squamous Cell Carcinoma | | | | | |
| Cases | 7 | 1 | 4 | 34 | 76 |
| RR | 1.0 | 5.49 (0.62, 48.4) | 5.45 (1.36, 21.9) | 15.73 (5.94, 41.7) | 22.1 (8.82, 55.2) |
| Small Cell Carcinoma | | | | | |
| Cases | 2 | 1 | 5 | 60 | 110 |
| RR | 1.0 | 8.59 (0.73, 101.7) | 10.29 (1.82, 58.2) | 51.9 (11.84, 227.3) | 77.0 (17.83, 332.3) |
| Adenocarcinoma | | | | | |
| Cases | 85 | 4 | 13 | 83 | 185 |
| RR | 1.0 | 0.83 (0.28, 2.43) | 0.78 (0.39, 1.56) | 2.43 (1.56, 3.78) | 5.04 (3.35, 7.60) |
| Large Cell Carcinoma | | | | | |
| Cases | 11 | 7 | | 19 | 23 |
| RR | 1.0 | 1.21 (0.33, 4.41) | | 2.26 (0.77, 6.68) | 3.57 (1.21, 10.60) |
| All Histologic Types | | | | | |
| Cases | 105 | 6 | 29 | 196 | 394 |
| RR | 1.0 | 1.03 (0.43, 2.46) | 1.33 (0.81, 2.16) | 4.08 (2.94, 5.65) | 7.29 (5.36, 9.92) |

Modified after: Comparison of Aspects of Smoking Among Four Histologic Types of Lung Cancer by Kenfield et.al (2008) [6]

## 1.2. IRS-1 and connection to PHIP

IRS-1 PHIP is the short form of the insulin receptor substrate-1 pleckstrin-homology domain interacting protein which is a protein described to have a role in the tumor progression of melanoma. [1,2]

The insulin-like growth factor-1 receptor (IGF-1 Receptor) is a receptor that belongs to the receptor tyrosine kinases (RTK) to which for example also VEGF or EGF receptors belong to. In total there are seven subfamilies of receptor tyrosine kinases. These receptors have a tyrosine kinase domain on the intracellular side of the receptor. Some of them also have a cysteine rich domain on the extracellular side and some have an immunoglobulin like domain. But unlike these others IGF-1 receptor is a tetramer, consisting of two transmembrane chains and two chains on the extracellular side of the cell. The transmembrane chains have tyrosine kinase domains and the chains on the extracellular side of the membrane both have cysteine rich domains. As the IGF-1 receptor is activated by IGF-1 it plays a vital role in the insulin pathway. [7]

When IGF-1 binds to the receptor, a rearrangement of the transmembrane chains is induced bringing two kinase domains in close proximity. Thereby the receptor is activated and cross-auto phosphorylation of the tyrosines in the tyrosine kinase domains enhances the kinase activity. Then the insulin receptor substrate-1 (IRS-1) which is associated with the insulin receptor (IR), is phosphorylated on multiple tyrosines which creates phosphotyrosine docking sites. Other intracellular signaling proteins use these docking sites to further process the signal and thereby making the metabolic and growth responses to IGF-1 possible. Thereby the IRS-1 (and also other insulin receptor substrate proteins) is a signaling adaptor protein. [7]

The IRS-1 has a pleckstrin homology (PH) domain at its N-terminus and also a phosphotyrosine-binding (PTB) domain. It binds to the IR using this PTB domain and the PH domain functions as a binding site for the pleckstrin-homology domain interacting protein (PHIP).[8,9]

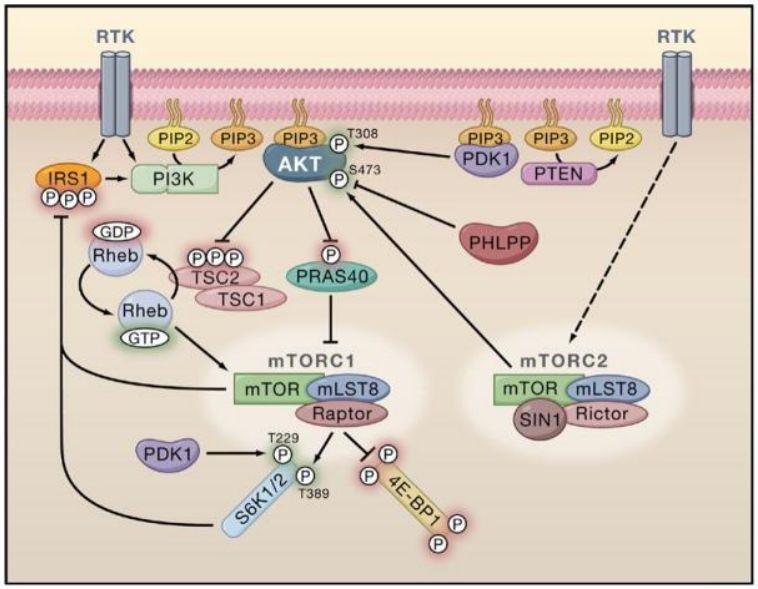
A recently discovered isoform of PHIP, PHIP1, containing a WD40 repeat and 2 bromodomains, 206kDa, was found to be involved in postnatal growth and translocation of the glucose transporter 4 (GLUT4), which plays a role in the insulin-pathway and in cardiac muscle cells. [1011]. Also this isoform of PHIP is predominantly found in the nucleus.

## 1.3. IRS-1 pathway in cancer

IRS proteins are cytoplasmic adaptor proteins that were first known for their role in the insulin-pathway. Thisfamily of proteins consists of 4 IRS protein (IRS-1-4). The predicted molecular weight of IRS-1 is 132kDa, but it has a lot of serine sites that are regularly phosphorylated before insulin stimulation, so its real size ranges from 165-170kDa. Upon insulin stimulation also tyrosine sites get phosphorylated which increases the size of IRS-1 again to 175-180kDa.[12]

As mentioned before, IRS-1 binds to the IGF1-receptor and gets activated by phosphorylation when ligand binding occurs. IGF are growth factors that promote survival of cancer cells. Proliferation, inhibition of apoptosis as well as angiogenesis and metastasis are only some of the events triggered by the signaling cascade leading to the activation of MAPK/ERK, AKT (protein kinase B) and PI3K (Phosphoinositid-3 kinases) pathways.[13]

**Figure 1**: Upstream activation of Akt and downstream effectors




from Manning, 2007 [14]

As stated previously, the IGF-1 receptor belongs to the RTK and PI3K is either activated by direct binding to IGF-1 receptor or through binding to the scaffold protein IRS-1.

Phosphorylation of PIP2 by PI3K generates PIP3 (PTEN can reverse this phosphorylation). AKT and PDK1 bind to PIP3 and AKT is activated by PDK1. The activated AKT activates mTORC1 over RHEP-GTP and will have positive effects on cell proliferation and growth. The mTORC2 complex is also activated by RTK-signaling pathway, which is not fully understood and mTORC2 can also activate AKT by phosphorylation.
So only pAKT is active in cells.

## 1.4. PHIP structure and family

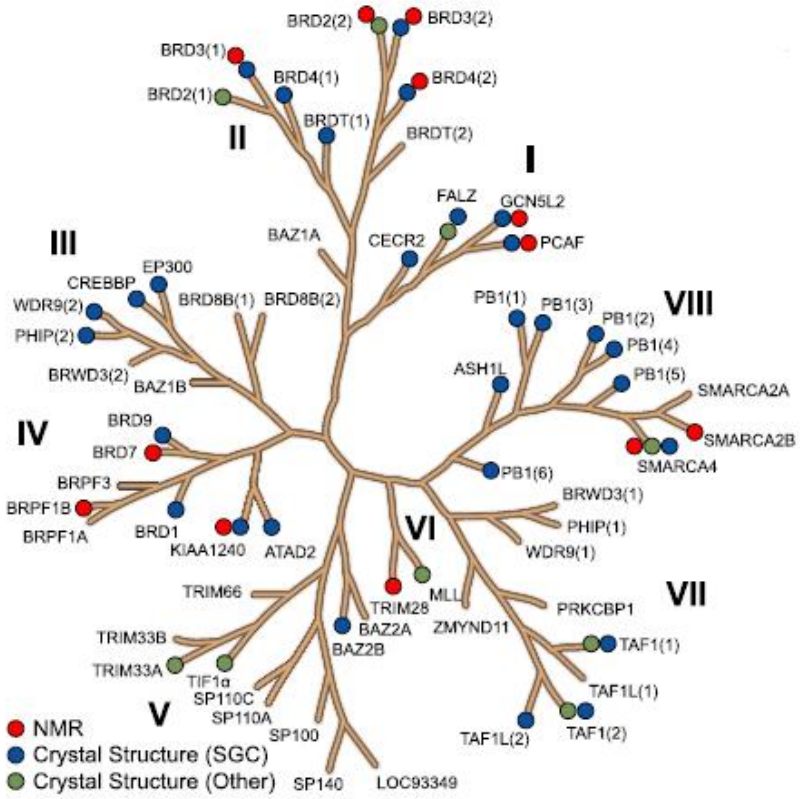
PHIP belongs to the family of bromodomain (BRD) containing proteins. Usually BRDs are present in nuclear proteins such as helicases or transcriptional coactivators. Bromodomains recognize ε-N-lysine acetylation motifs and bind to them. Proteins having a BRD can be grouped into eight families according to their structure or sequence similarity, covering all 61 BRDs that can be found in the human genome.[15]

ε-N-acetylation of lysine residues describes the posttranslational addition of an acetyl group to the ε-amino group, which is positively charged, of the amino acid lysine, neutralizing the positive charge.[16] The acetylation level of proteins is controlled by histone acetyltransferases and histone deacetylases and is considered to be the most abundant posttranslational modification of proteins. [17,18]
Interestingly, BRD can be found quite frequently in larger proteins that function in DNA damage, chromatin-remodeling or that play a role in cell cycle control, and this might already give some hints about the possible role of PHIP in cancer and the exact pathways leading to the effects that overexpression of PHIP has on cells.
Binding specificity of BRD proteins is achieved by a conserved region shared by all BRD proteins, which consists out of four α-helices. [15]

A dysfunctional (due to mutations and changes in the protein sequence) bromodomain of a protein can be the cause for multiple diseases. For example a chromosomal

translocation leads to the fusion of BRD4 and BRD3 and NUT which leads to a very aggressive form of squamous cell carcinoma.[19]

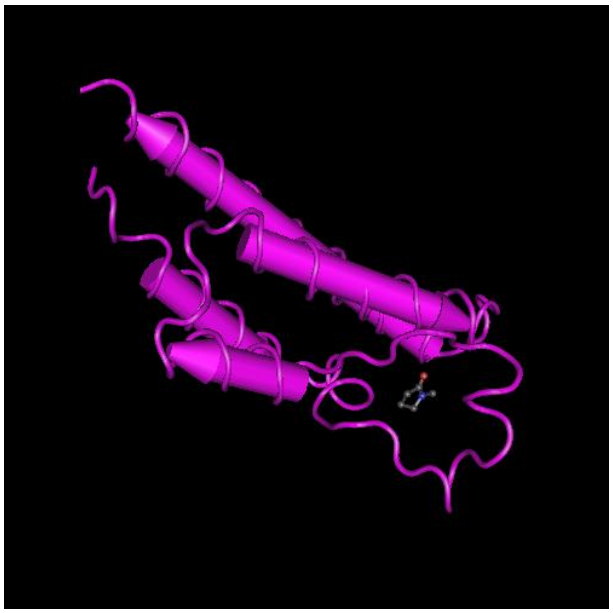
**Figure 2**: Phylogenetic Tree of the Bromodomain Family, the blue, green and red dots identify the different structures identified. PHIP1 and PHIP2 indicate the two different bromodomains of the PHIP protein.




Modified from Filippakopoulos et al., 2011[15]

As shown in Figure 2, PHIP belongs to the bromodomain family III and is closely related to BRWD1 and BRWD3 which play an important role in leukemia and WDR9. Both belong to the family of the WD repeat family. Members of this protein family play a role in apoptosis, cell cycle progression, gene regulation and others. PHIP, BRWD1 and BRWD3 have two bromodomains and WD40 repeats in common.[20] Figure 2 and 3 also indicate that the crystal structure of PHIP bromodomain has been described.

**Figure 3**: Crystal Structure of the Second Bromodomain of PHIP which was found to be druggable



Adapted from Madej T.,2014 [21]

## 1.5. PHIP in cancer

PHIP is a signaling protein which is transmitting a signal stemming from ligand binding into the interior of the cell by binding to another protein. This thesis is dealing with the insulin pathway in connection to PHIP but PHIP is also participating in some others pathways discussed in this thesis.

Before 2012, PHIP was only reported to be involved in the Insulin pathway and thereby helping to pass on the signals which are leading to the metabolic and growth responses of insulin[10]. It was also found that PHIP is participating in postnatal growth.[11] In 2012, De Semir D et al. first described a role for PHIP in the progression melanoma. For the role of PHIP in cancer it is important to know that PHIP is overexpressed especially in metastasized melanomas and is also having a high copy number in these cells when compared to the amount of expression in primary tumors. This observations made PHIP a prognostic marker.

The theoretical idea was that tumors over-expressing PHIP are prone to metastasize and, as suggested by another study of this research group, also prone to ulceration,

which means an overall worse condition for the patient compared to a melanoma that is not overexpressing PHIP[2]. To identify the exact role of PHIP in the progression of cancer (melanoma in this case) and to prove this theorem, PHIP mRNA was targeted with the help of a siRNA and a drastic reduction of the RNA expression was the outcome, which, in turn, also leads to a reduction in protein levels.
Transfection of melanoma cells with the same shRNA construct also showed reduction in their invasive capability, meaning that PHIP can directly be linked to invasive activity of melanoma cells.
To prove the effects of PHIP also *in vivo* the shRNA-expressing mouse cells were injected into mice by tail-vein injection and as a result there where less lung nodules found in these mice when compared to a control group. Also when injecting melanoma cells that where infected with the anti-PHIP shRNA into mice they were able to survive for a prolonged period of time when compared to control mice.[1]

Another study performed by this group is linking PHIP to the amount of ulceration in melanoma and also suggesting it as a prognostic marker. According to this study high PHIP copy numbers (analyzed by fluorescence in situ hybridization-FISH) goes hand in hand with not only a higher level of expression, but also a decreased distant metastasis-free survival, which is the time until metastasis occurs or can be detected. Furthermore a high copy number of PHIP also leads to a higher risk of ulceration, which is the breakage of the skin, caused by a melanoma and is an important prognostic factor as well as a hint on how well the patient might respond to a specific treatment. The cause of ulceration is known to be linked to elevated vascularity in the tumor but statistical analysis revealed that the level of PHIP copy number is also predictive for ulceration, independently on other factors, such as the tumor vascularity or the dimensions of the tumor and its mitotic rate but the exact biological pathway is poorly understood. [2]

As very little is known about the exact pathways PHIP is playing a role in, it is important to assess the changes PHIP is having on the cells' main pathways that will ultimately lead to effects as increased invasive ability of melanoma cells and their metastatic potential. Because PHIP is mainly interacting with the Insulin pathway (IGF-1 pathway

in specific) the idea that the metabolic pathway is going to be influenced by different levels of PHIP is obvious.

When introducing the same shRNA in melanoma cells as in the previous stated project [1] targeting PHIP, they had a reduced glycolysis metabolism as evidenced by the lower levels of lactate produced than a control cell line, resulting from lower levels of lactate dehydrogenase. Lactate dehydrogenase is catalyzing the step in the lactic acid fermentation in which pyruvate is converted to lactate.

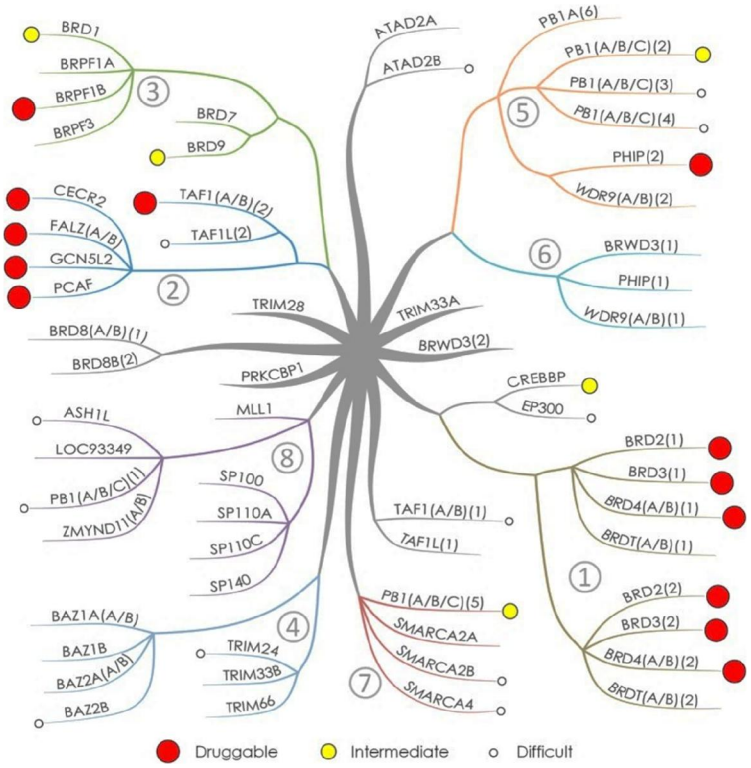
But when these cells are cultured in media containing methyl-pyruvate their invasive capability is increased. Pyruvate is the result of glycolysis and then enters the Krebs cycle, so by adding methyl-pyruvate to the media, glycolysis can be bypassed which is a proof that PHIP is increasing the invasive potential of cells, at least to some extent by activating the glycolytic pathways.

Another important factor to be controlled by PHIP is the vascular endothelial growth factor (VEGF) which was found to be lower in melanoma cells expressing the PHIP shRNA construct. This finding is backed up by a western blot showing lower levels of the hypoxia-inducible factor 1 alpha subunit (HIF1A) in PHIP suppressed cells.

VEGF is an important factor regulating vasculogenesis and angiogenesis promoting tumor growth. To prove this effect *in vivo* melanoma cells were injected subcutaneously into mice and although the tumor volume was the same the anti-PHIP shRNA cells were causing necrosis due to smaller blood vessels and a lower microvessel density.[2]

Computational analysis have shown that PHIP is druggable which makes it a target for cancer therapy by specific inhibitors (see Figure 4)[22]. In fact, a first low affinity inhibitor for PHIP bromodomain has been reported.[23]

**Figure 4**: Bromodomain classification tree showing druggable bromodomains (red dots)




Adapted from Vidler LR et al 2012.[22]

Because of these proliferation and invasion effects on melanoma progression due to the modulation of PHIP expression, we wanted to assess the broad-based role of PHIP in other cancers, namely lung cancer.

# 2. Materials and Methods

First of all a lentivirus containing an anti-PHIP shRNA and a selectable marker gene as well as a control virus targeting an irrelevant gene will be generated and used to stably infect the lung cancer cells.

The cells that have integrated the desired gene are selected with the help of the marker gene and a specific drug and the expression of the gene of interest is confirmed via quantitative RT-PCR.

When gene knockdown is confirmed protein levels are determined by western-blot electrophoresis and also by quantitative immunofluorescence.

As mentioned before the reduction of the protein should lead to a change in cell behavior which will be studied by cell cycle analysis and other assays.

## 2.1 Lentiviral generation and infection of cells

The 7084bp long pLKO.1 hairpin vector was digested with AgeI and ECORI. To create the control knockdown cell line the shRNA targeting luciferase (luc) with the DNA sequence 5'- CGTACGCGGAATACTTCGATTCAAGAGATCGAAGTATTCCGCGTACG -3' was ligated into the vector. For the PHIP Knockdown stables the same lentiviral vector construct was used and the shRNA TRCN0000130419 that targets the human PHIP mRNA at position 5436 with the sequence 5'-CCGGGTTAAGGAGAAGTAACCGAAACTCGAGTTTCGGTTACTTCTCCTTAACTTTTTTG-3'. Additionally another anti-PHIP shRNA was proven to target PHIP. TRCN0000127738 with the sequence CCGGGCTGGAAGACAGTCTTTACTACTCGAGTAGTAAAGACTGTCTTCCAGCTTTTTTG-3' targets the mRNA at bp 532. Both shRNAs stem from a set of shRNAs (Openbiosystems) all designed to target PHIP but these two gave the best knockdown results and where thus used to work with. The lentivirus was prepared using a three-plasmid packaging system using a pLKO1-puro vector.

293T cells were transfected using a lipofectamine protocol.

After 48 hours the lentiviruses in the supernatant can be harvested and quantified.

H1703 and Calu-3 cells were then infected with the titrated virus with the help of 8 µg/ml

polybrene in order to get a higher success rate. After three days selection using puromycin began.

## 2.2 Taqman

### 2.2.1 RNA extraction

For the extraction of the RNA from a cell pellet the RNeasy MiniKit (Quiagen, Cat# 74106) was used as described in the manufacture manual.

First 10µl ß-mercaptoethanol (Sigma Aldrich, Cat# M3148-25ML) were added to every 1 ml of the RLT lysis buffer to get rid of RNAses in the sample. 350µl of this mixture are added to te pellet and resuspended into a QIAshredder spin column (Qiagen, Cat# 79656) and centrifuged at full speed for two minutes in a table centrifuge. 350 µl of 70% ethanol was then added to the flow through and the mixture transferred to an RNeasy Mini spin column which was then centrifuged for 15 seconds at 8000g. As the mRNA will be retarded by the filter the flow-through can be discarded and 700µl of RW1 buffer was added to the column and it was again centrifuged with the same parameters as in the previous step.

The flow-through was discarded and 500µl RPE buffer added in order to increase purity of the sample while centrifuging. As this is an important step in this procedure it is repeated, so the flow-through is discarded and RPE buffer is added and the column centrifuged for 2 min at 8000g. After discarding the flow-through the column is placed into a new 2 ml collection tube and centrifuged for 1 minute at full speed which will dry the membrane. After this the column is then placed into a new 1.5ml collection tube (Eppendorf) and 30-50µl (depending on pellet size) RNase –free water was added. One minute was given to allow the water to soak the membrane, followed by a final centrifugation step of 1 minute at full speed. From now on the tube has to be stored on ice as the RNA in the collection tube is temperature sensitive.

The concentration and purity of the RNA was then measured with a NanoDrop 2000 UV-Vis Spectrophotometer, where special attention was paid to the 260/230 and 260/280 values as RNA that is impure cannot be used for further analysis and has to be purified by ethanol precipitation.

### 2.2.2 cDNA synthesis:

For the synthesis of RNA to cDNA a specific amount of RNA together with RNase free water as well as a reaction mix (or buffer) containing primers, the DNA polymerase, dNTP's and oligos as well as other ingredients that stabilize the reaction. The last ingredient for this reaction is the reverse transcriptase (RT) enzyme. All these ingredients are available in the iScript cDNA synthesis Kit (BioRad, Cat# 170-8891)

First the needed amount of RNA (1µg per reaction) has to be calculated using the concentration. Then the needed volume of RNA was made up to 7.5µl with RNase free water. To this mix 2µl buffer and 0.5µl RT was added. As these are very small volumes the buffer and the RT can be mixed together separately for every reaction needed and then 2.5µl of the well mixed sample pipetted to every reaction in order to avoid pipetting errors stemming from small sample volumes. The whole reaction is pipetted into small PCR tubes which should be stored in ice together with the individual ingredients as the reaction is temperature sensitive.

The PCR reaction runs in the PCR machine in the following program:

25°C – 5 min

42°C – 30 min

85°C – 5 min

4°C – until further use (long term storage at -20°C)

### 2.2.3 qRT-PCR with TaqMan probes

For the PCR PCR reaction every sample was added in triplicates to a fast optimum 96-well plate and at least two probes (housekeeping/control gene and gene of interest/GOI) per cDNA sample were used to obtain reliable results.

First the cDNA was diluted in a ratio of 1:5 with Nuclease free water. The reaction mix itself contains the diluted cDNA, nuclease free water and the "Fast advance mastermix "(Applied Biosystems, Cat# 4444557). The volumes of the ingredients are shown in the table below and are calculated for the use of 1.7µl of a Taqman probe per sample. This means that per cDNA sample the reaction mix was set up in one Eppendorf tube as shown below and then depending on whether 2 or 3 probes were used, the reaction mix

was either split up into new Eppendorf tubes in duplicates or triplicates of 34µl each and 1.7µl of the respective TaqMan probe was added to every tube. It is important to make sure that every mixture is mixed before distributing it. This is especially crucial when adding the samples in triplicates of 11µl each into the 96-well plate to make sure every reaction is comparable to another. Furthermore the whole experiment should again be performed on ice to avoid a change in the quality of the ingredients. To avoid vaporization of the samples the plate was covered with a sealing film and the run was performed in a 7500 Fast RT-PCR system.
For this project the housekeeping gene used for the negative control was Ubiquitin C (UBC).

## 2.3 Western Blot

In order to extract the proteins out of the collected cell pellet was resuspended in 50-75µl RIPA lysis buffer (Thermo Scientific, Prod# 89900) to which a 100x Protease and Phosphatase inhibitor (HALT, from Thermo Scientific, Prod# 1861281) was previously added in order to dilute it to 1x. The resuspended mixture was then added to a new Eppendorf tube and with the help of a syringe the cells where carefully lysed. The tube was then placed on ice for 30 minutes and after that centrifuged for 10 minutes at maximum speed and attention has to be paid that the centrifuge is cooled down to 4°C. The supernatant is transferred to a new Eppendorf tube and is either kept on ice if used directly or stored in -20°C is not used immediately.

### 2.3.1 Protein quantification via Bradford assay

The samples were always measured in triplicates in order to get more reliable results. 160µl HBS and 40µl of the Bradford reagent (Bio Rad, Cat# 500-0006) were added per well and also to the wells for the standards. Then 1µl of the samples were added to the mixture and every sample was distributed evenly in the well. The standard curve was prepared by a serial dilution of 40µg, 20µg, 10µg, 5µg, 2.5µg and 0µg of BSA in duplicates. The concentration of the samples was measured via a spectrophotometer multiwall plate reader and the SoftMax Pro software.

### 2.3.2 SDS-Page

First the Gel to separate the proteins has to be prepared according to the following table.

**Table 2**: Composition of separating gels in different acrylamide concentrations

| | 5% | 7.5% | 15% |
|---|---|---|---|
| $H_20$ | 5.7 | 5.1 | 3.5 |
| 40% acrylamide | 1.125 | 1.7 | 3.375 |
| 1.5M tris | 2 | 2 | 2 |
| 10%SDS | 80ul | 80ul | 80ul |
| APS | 75ul | 75ul | 50ul |
| TEMED | 8 | 8 | 8 |

**Table 3**: composition of the stacking gel (4%)

| | |
|---|---|
| $H_20$ | 3.1 |
| 40% acrylamide | 0.5 |
| 0.5 M tris-glycine | 1.25 |
| 10%SDS | 55ul |
| APS | 50ul |
| TEMED | 5ul |

First the separating gel has to be filled into the tray, and after this one solidifies the stacking gel has to be filled.

Then the proteins need to be prepared for the loading step. Therefore the amount of protein has to be calculated and has to be mixed with loading dye (NuPage LDS Sample Buffer from Invitrogen, Cat# NP0007). The volume of dye added to the protein is ¼ of the volume of protein needed. This mixture is then heated in a heating block for 5 minutes at 95°C.

The samples as well as the protein marker (Page Ruler plus prestained from BioRad, Cat#26619) were loaded into the wells of the solidified gel. The gel in the tray has to be covered with 1x running buffer (TGS, 10x from BioRad, Cat#161-0772). An electrical field between (80 and 120 V) was applied and the experiment was run until the bands of interest were well separated.

### 2.3.3 Protein Transfer

There are two different methods of protein transfer that were used during this project. Wet transfer was used for larger proteins and semi-dry transfer for smaller proteins. For the semi-dry transfer the gel and the nitrocellulose membrane were placed in between 4 filter papers (2 on each side) and Transfer Buffer (1x TGS containing 30% MetOH) was used to make sure that every layer is wet, as well as the contact plates of the machine. The transfer was running at 25V for 15 minutes.

For the wet transfer the gel and the membrane were covered with a filter paper and a sponge on each side and were put into a cassette. The cassette was filled with transfer buffer and an ice tray to avoid overheating of the buffer. The run was performed in the cold room, another measure to avoid overheating, for 1 hour at 100V, or 2h at 75V, depending on the size of the protein of interest.

### 2.3.4 Antibodies and development

When finishing the transfer the membrane was blocked for 30 minutes in 5% milk made out of milk powder in 1xTBST (TBS,10x, Corning; Cat# 46-012CM), (Tween-20, Santa Cruz Cat# sc-29113).The membrane was then put into a plastic pouch into which the corresponding primary antibody was added in the correct dilution (provided by supplier) in 5% milk in TBST. The pouch was then sealed and left on the shaker overnight in the cold room. In the morning three 10 minute washes with TBST were performed and the corresponding secondary Antibody was added in a 1:200 dilution in TBST and left on

the shaker at room temperature for one hour. The secondary antibodies used were either anti-mouse (BioRad, Cat#170-6516) or anti-rabbit (BioRad, Cat# 172-1019) depending on the primary antibody. After three 10 minute washes the "Western Blotting Luminol Reagent" (Santa Cruz Biotechnology, Cat# sc-2048) was added to the membrane. This is the final step and the membrane is now ready to be developed.

### 2.4 Immunofluorescence staining

First the cell line to be tested was plated on cover glass slips that were placed in 6 well plates and were grown under normal conditions until they were confluent. Then the media was removed and the wells were washed three times with PBS (Fisher Scientific, Cat# F79-500) to make sure that no remaining media is left. Then they have to be fixed immediately with 4% Formaldehyde diluted in PBS for 10 minutes on a shaker. Following three PBS washes 0.2% Triton X-100 (MP Biomedicals Cat# 807426) diluted in PBS was added for 10 minutes on the shaker. Again three washing steps were performed and 3% BSA (Albumin, from bovine serum; Sigma-Aldrich, Cat# A7906-100G) diluted in PBS was added for 10 minutes on the shaker. After three more washes with PBS the desired primary antibody diluted in 250µl Antibody diluent (Dako, Cat# S3022) was added to the corresponding wells and the plate was incubated overnight at 4°C. Following three PBS washing steps the correct secondary antibody was added in a dilution of 1:1000 in 500µl of the antibody diluent and the plate was incubated for one hour at room temperature in the dark. There were four different antibodies used for the immunofluorescence staining, all from life technologies: Mouse red or green (Cat# A11020 or A11017) and Rabbit red or green (Cat# A11072 or A11070). After three washing steps the slides are ready to be mounted on microscope glass slides. Before the mounting step the slides were dehydrated with the help of an ethanol series (70%, 90%, 100% EtOH). Then the slides were mounted with the help of Vectashield mounting medium containing DAPI (Vector Laboratories, Cat# H-1200). After this final steps the slides were ready to be examined under the electron microscope where it is important to note which protein will appear in which colour (depending on secondary antibody). It is crucial for all the washing steps to lift the cover slip each time in order get rid of any remaining impurities.

## 2.5 Cell survival assay

For the cell survival assay 5000 cells were plated per well in a 96-well plate and were grown in the respective culture media containing puromycin for 4 days, whereas every time point is measured in quarduplicates. The absorbance of cells was measured every day at the same time point by adding 10 µl of the Dojindo cell counting kit-8 (Dojindo, 500 tests, # CK04) and reading the plate in a spectrophotometric multiwell plate reader at 450nm. As the wells that were already treated with Dojindo cannot be measured the next day again 16 wells in total per knockdwown cell line were needed to get reliable results.

## 2.6 Colony formation Assay

3000 cells were seeded in 6 well plates with the corresponding growth medium containing puromycin and were incubated under normal conditions for 6 to 10 days. After colonies were seen with the bare eye the medium was removed and the colonies were stained with crystal violet solution (Sigma Aldrich, Cat# HT90132-1L) for 10 minutes and the wells were then washed with water until colonies can be counted.

## 2.7 Invasion assay

### 2.7.1 Coating and loading of cells:

This experiment is carried out in 24-well plates containing special inserts (Corning, Prod# 10274901) First the chambers with the filter on the bottom (called upper chamber) were placed into the wells of a 24 well-plate (called lower chamber). The upper chamber must be coated with matrigel (Corning, Prod #354230), therefore matrigel was diluted in the desired concentration in the appropriate media (without FBS). Next the matrigel was added onto every filter and distributed evenly, so that the thickness of the coat is equal all over the membrane. The amount of matrigel used in this step determines the overall thickness of the coating but is usually 15µl. After 45 minutes at RT the matrigel solidifies and the cells can be added.

The harvested cells were counted using a counting chamber and were diluted so that approximately 150 000 cells per 200µl media (without FBS) were placed into every upper chamber. In the lower chamber 300µl of the proper media containing 20%FBS were pipetted in. It is important that there are no bubbles between the two chambers, if so, the upper chamber has to be readjusted. The plate was then incubated ON in the incubator.

### 2.7.2 Fixation, staining, counting

Before taking the plate out of the incubator a 2.5% glutaraldehyde in PBS solution and a 0.5%toluidine blue/2% Na2CO3 in ddH20 solution were prepared.
After that the media from both chambers was removed and 500µl of the glutaraldehyde solution was filled into each lower chamber. Then PBS was filled into new (empty) wells of the 24-well plate and the upper chambers were transferred to these wells containing PBS only. Then the PBS was removed and 500µl of the toluidine blue/Na2CO3 solution was filled into these wells and was incubated for 5 minutes at RT.
After these 5 minutes the upper chamber gets “washed” in a beaker containing PBS only. This washing step was performed by carefully dipping the chamber into the beaker several times using a forceps. To remove the cells that did not migrate through the matrigel and filter to top of the filter was carefully wiped several times with a cotton cutips. The chambers were placed back into empty wells and cells were counted under the microscope.

## 2.8 In vivo experiment

For the in vivo tumor growth experiment the cells to be injected into the ice first had to be grown in a large quantity to have enough cells to be injected into the mice. This is carried out in multiple p150 plates in the corresponding growth medium containing puromycin. The cells need to be trypsinized and counted before injection by using a hemocytometer. Then 5 million cells per mouse need to be injected subcutaneous into nude and immunodeficient mice. These 5 million cells were injected in a volume of 100µl, consisting to 50% of matrigel and to 50% of RPMI only. The matrigel is providing

a basis for the cells to grow in the in vivo environment and to adjust to it. The left flank of the mice was chosen as a site for injection.

For this experiment only 6 mice could be injected with H1703 127 because there were not enough cells to inject. Gladly there were enough H1703 luc cells cultured and 10 mice could be injected with these cells.

The mice are kept in accordance to the guidelines for animal studies and the tumor volume is measured regularly using a caliper. The measurement is carried out double-blinded to avoid bias and a change of the size of the tumor.

For this experiment the mice were kept alive as long as it was ethically justifiable, and where then euthanized according to guidelines and protocols established.

The statistical calculation of the tumor volume using the output of the tumor measurement is carried-out using the formula $\frac{Length*width^2}{2}$.

## 2.9 Basic cell culture

H1703 cells were cultured in cell culture plates having a diameter of 100 (p100). They were grown in RPMI-1640 medium (Corning cellgro, Prod# 26616006) containing 10% FBS (JRScientific; Cat# 43640-500) and under constant penicillin/streptomycin treatment. (P/S, GE Healthcare Life Sciences, Cat# SV30010). As mentioned before they were also under constant puromycin selection to maintain the vector and therefore the downregulation of the target proteins. For this purpose puromycin (Thermo scientific, Cat# A1113802) was added to the respective plates after each splitting process in a concentration of 0.5µg/ml.

Cells were constantly kept in a sterile environment in a cell culture incubator providing a temperature of 37% and a 5% CO2 concentration.

### 2.9.1 Splitting the cells:

The cells were examined and checked every second day under a light microscope to detect possible bacterial infection and also to estimate the best time point for splitting the cells. H1703 luc and 127 cells were split at 80% confluency, which happened to be every second or fourth day. For the splitting process the media was first removed from

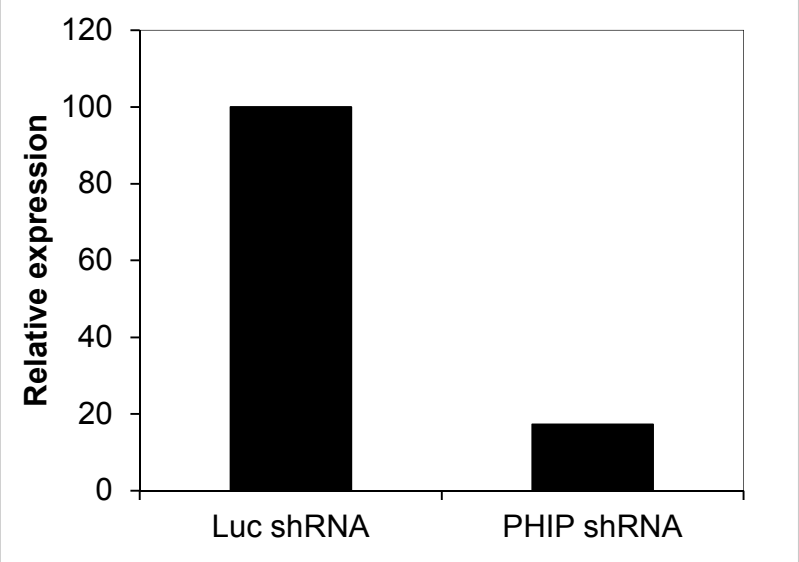
the plate which was afterwards rinsed with PBS. After a short incubation with 1ml Trypsin (HyClone (GE Life Sciences, Cat# SH3004201) in the incubator. When detachment of cells can be seen under the microscope, 5 ml prewarmed media containing FBS and P/S were added to stop the reaction. A homogenous mixture of the cells in the media is achieved by pipetting carefully and splitting is performed by removing 5 ml of the solution and discarding it appropriately. 9 ml of the media is added again as the cell culture plates need to have a volume of 10 ml for optimal growth of the cells. Additionally the correct volume of puromycin has to be added.

# 3. Results

## 3.1. Taqman

In order to continue to work with the generated PHIP knockdown cell lines and to examine their behavior as discussed previously, the gene or mRNA knockdown has to be confirmed. mRNA knockdown means the amount of a specific mRNA, in this case the PHIP mRNA expressed in a cell line at a specific time point is lower than in the control cell line. This knockdown is achieved by culturing the cells in media containing puromycin or any other antibiotic to which the cells required resistance by infection with a plasmid. The optimal puromycin concentration was tested previously by treating the parental cell line with different levels of puromycin and estimating cell survival.
As a negative control the housekeeping gene UBC was used for the Taqman analysis.

**Figure 4:** relative expression of PHIP mRNA in H1703 luc vs H1703 127.




The graph clearly shows that cells stably infected with the PHIP shRNA express lower levels of PHIP mRNA compared to the control cell line expressing luc shRNA. The PHIP mRNA levels of the luc shRNA infected H1703 cells are considered as 100%. The expression levels of PHIP in the knockdown cells have a level of 16% in H1703, the levels for the Calu-3 cell line can be seen in the supportive data. The standard deviation

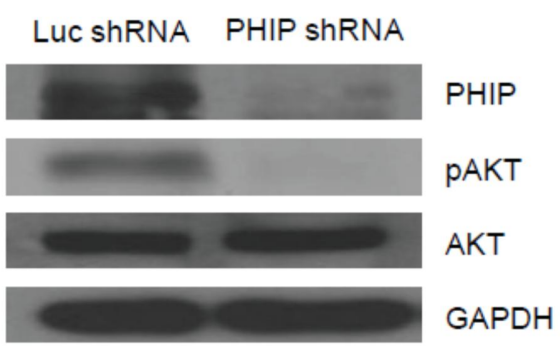
of three measurements of H1703 127 tested for PHIP is 0.066 and when testing the cDNA for UBC, the control gene used, the standard deviation is 0.056.

The 7500 Fast RT-qPCR machine is giving the operator an output showing the probes and their corresponding threshold cycle ($C_T$ – values). The threshold cycle is the intersection of a set threshold value and the amplification curve of every single sample.
An average $C_T$ for the triplicates of every sample for every probe has to be calculated first. Then the difference in $C_T$ between the housekeeping/control gene and the gene of interest (GOI) is calculated by substracting the $C_T$ of the GOI from the $C_T$ of the housekeeping gene. After this the relative expression normalized to the housekeeping gene was calculated using the formula $\%_{rel.Expression} = 2^{-dC_T} * 100$. The Knockdown in percent is calculated with the help of the formula $\%_{Knockdown} = 100 - (\frac{\%_{rel.Expression\ GOI\ in\ Knockdown}}{\%_{rel.Expression\ GOI\ in\ control}} * 100$, and finally to plot the bars the percentage of GOI expression in the two subjects is calculated by substracting the percent of expression from 100.

### 3.2. Western Blot

To also confirm knockdown of PHIP at protein levels a Western Blot was performed. In order to make sure that the obtained results are true the membrane was also tested for other proteins that should not be affected by the knockdown of the PHIP gene.
To exclude a wrong interpretation by the human examiner the pixel-intensity of the bands were also quantified using the software ImajeJ but the numbers are not shown here as the result is pretty obvious.

**Figure 5:** H1703 Western Blot testing for Protein levels of PHIP and pAKT as well as for AKT, and GAPDH



PHIP as well as phospho-AKT is significantly reduced or nearly not visible at all which means that the corresponding protein levels are really low or not present at all. The expression of the control proteins Glycerinaldehyde-3-phosphate (GAPDH) and protein-kinase B (AKT) on the other hand is the same in both cell lines.

### 3.3. Immunofluorescence staining

Antoher way of quantifiying the proteins in the cell is to perform IF on them. As mentioned before the proteins of interest can be stained in different colors and the level of expression measured by a computer program. Furthermore the location of the protein of interest can be determined. To do so the different pictures are overlapped and if a yellow color can be observed it means that the protein is for example located in the nucleus.

A high intensity means that the specific protein the slide is stained for is present in a high amount.

**Table 4:** IF images for H1703 luc and H1703 127 cells stained for PHIP, IntegrinB1 and Dapi; The first column is showing the combination of all stainings; Results obtained by usage of a confocal light microscope

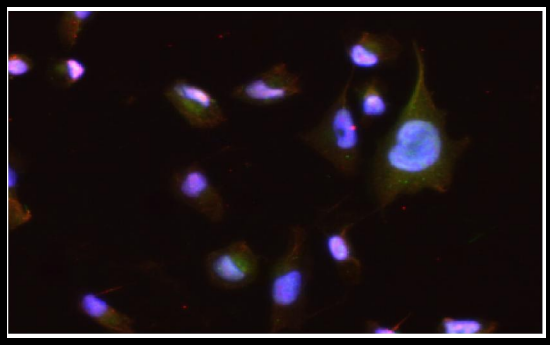
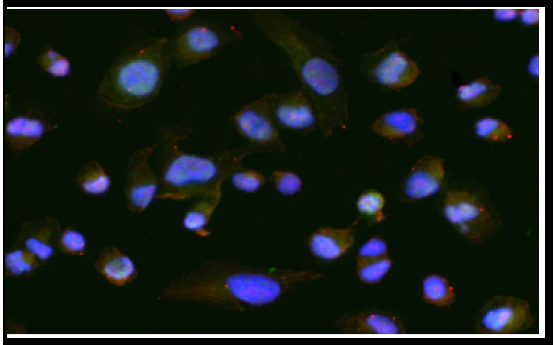
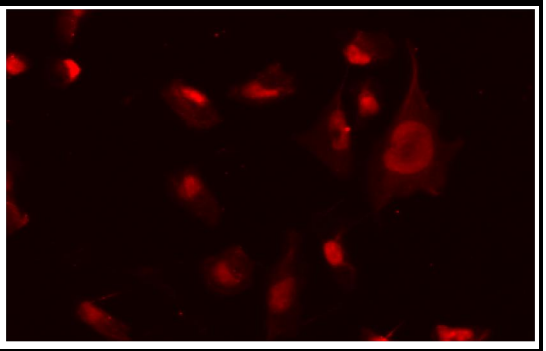
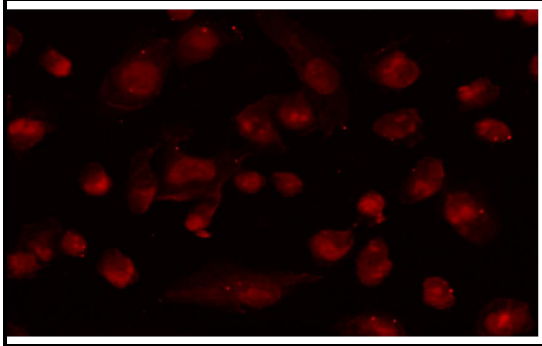
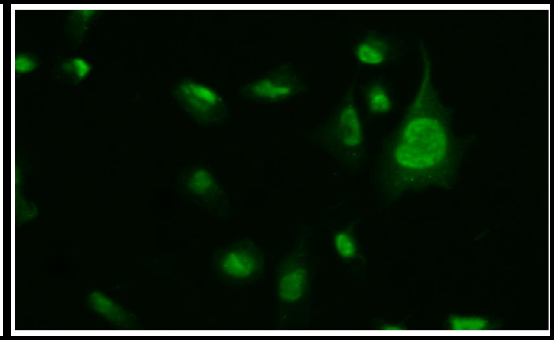
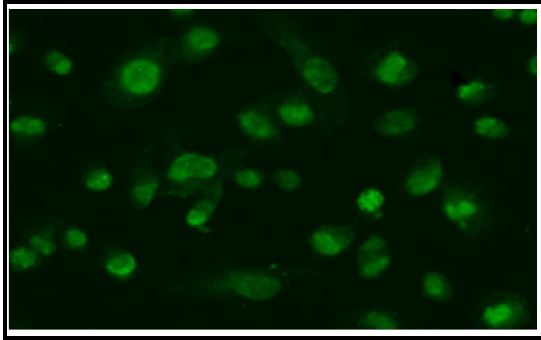
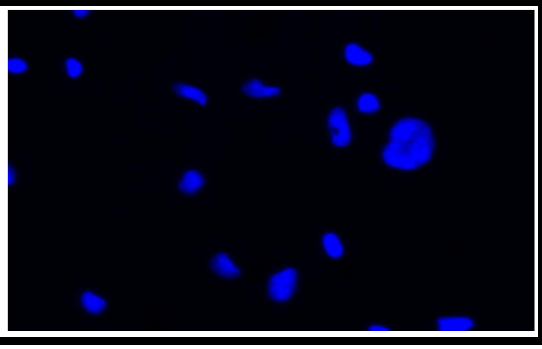
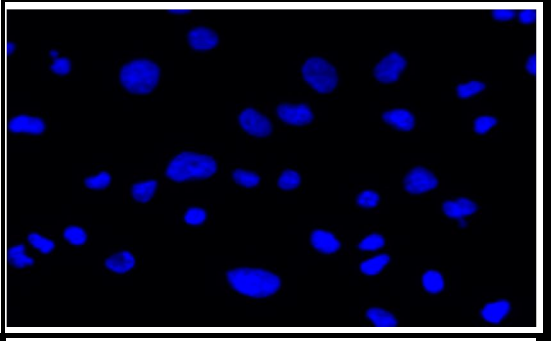
| **Protein tested for** | **H1703 luc** | **H1703 127** |
|---|---|---|
| Merge |  |  |
| PHIP |  |  |
| Integrin B1 |  |  |
| DAPI |  |  |

**Table 5:** IF images for H1703 luc and H1703 127 cells stained for PHIP, Cyclin D1 and Dapi; The first column is showing the combination of all stainings; Results obtained by a fluorescent microscope.

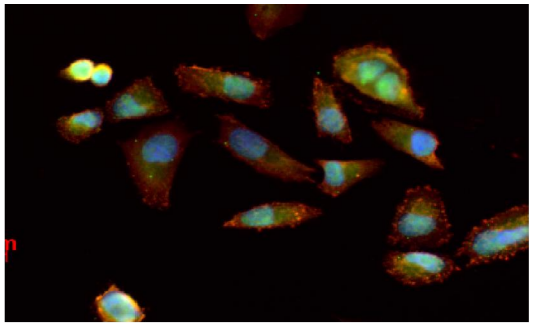
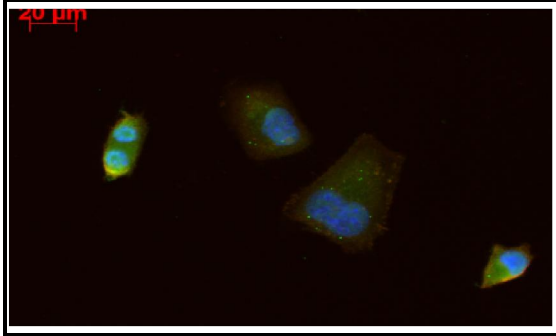
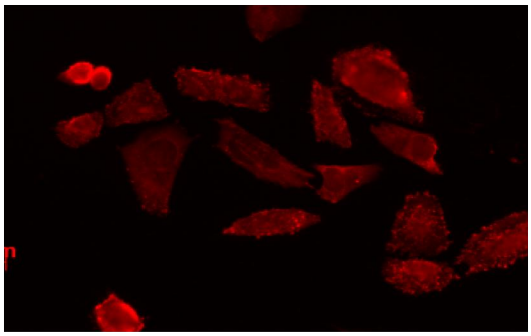
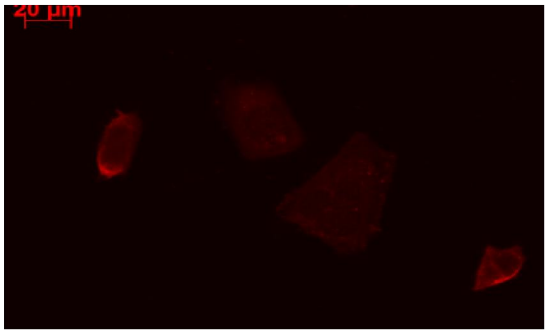
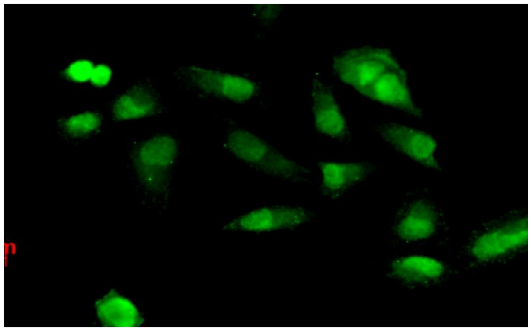
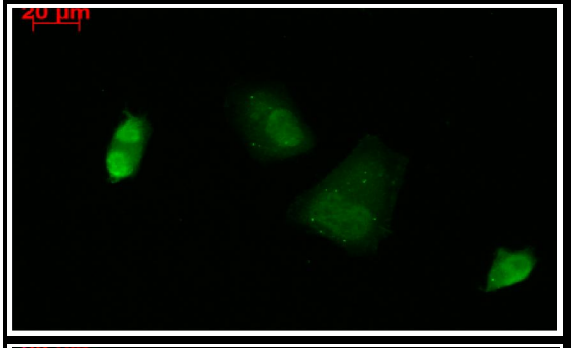
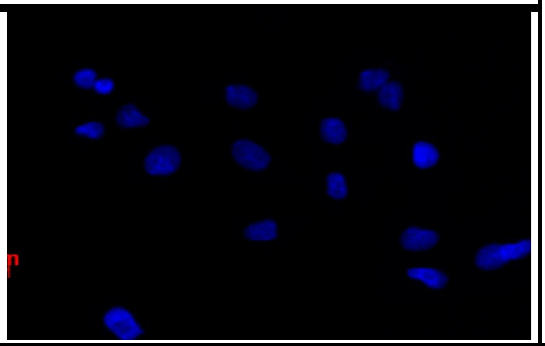
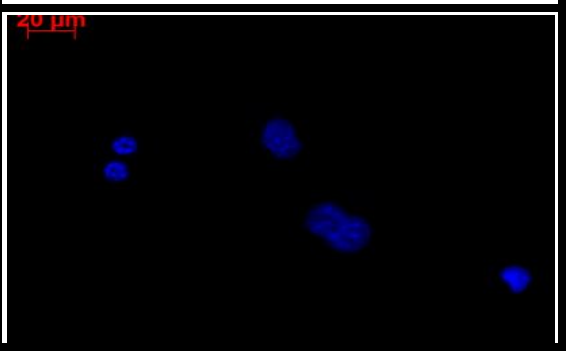
| Protein tested for | H1703 luc | H1703 127 |
|---|---|---|
| Merge |  |  |
| PHIP |  |  |
| CYCLIN D1 |  |  |
| DAPI |  |  |

**Table 6:** IF images for H1703 luc and H1703 127 cells stained for PHIP, Talin1 and Dapi; The first column is showing the combination of all stainings; Results obtained by a fluorescent microscope

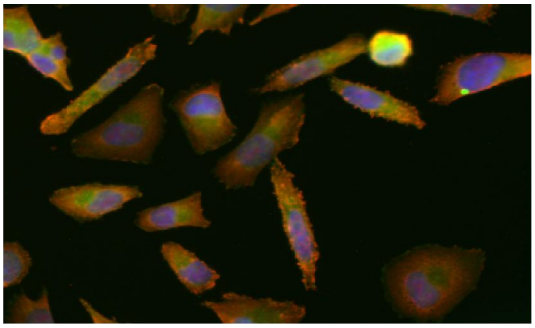
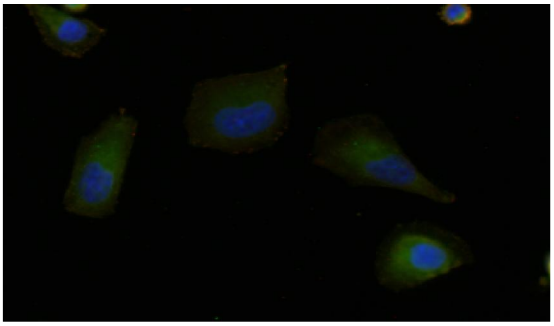
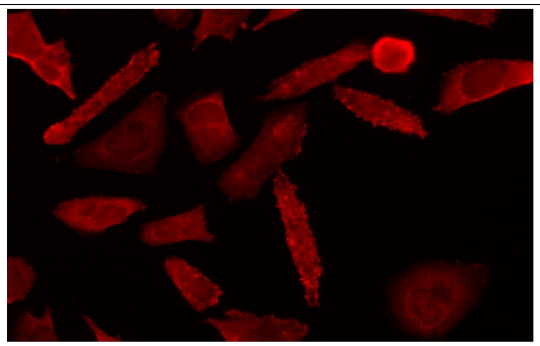
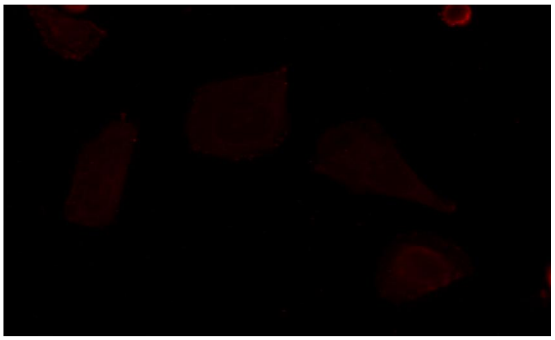
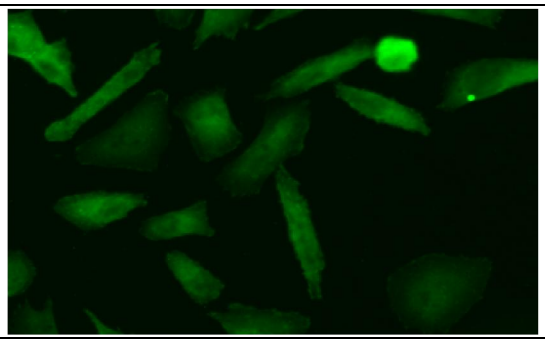
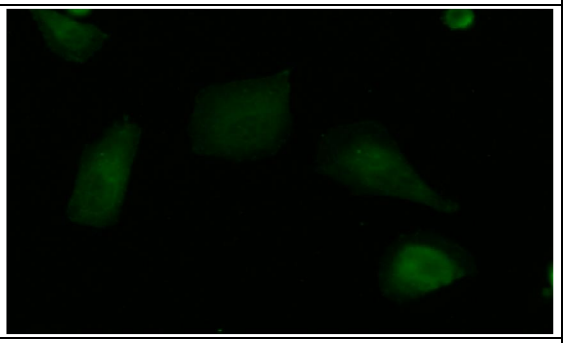
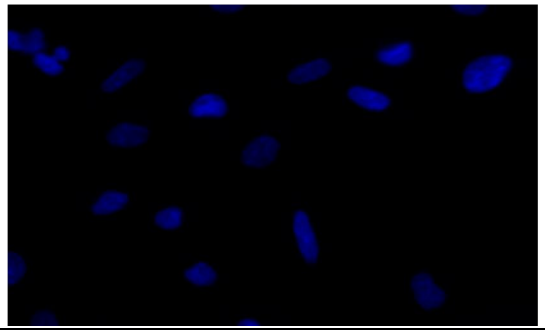
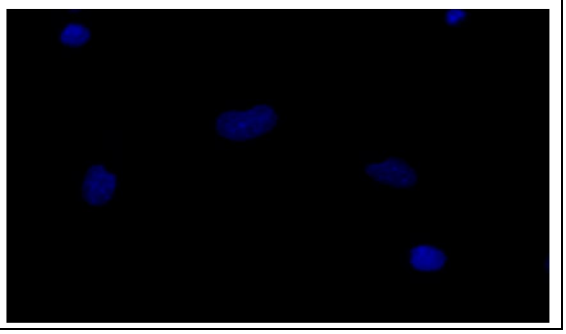
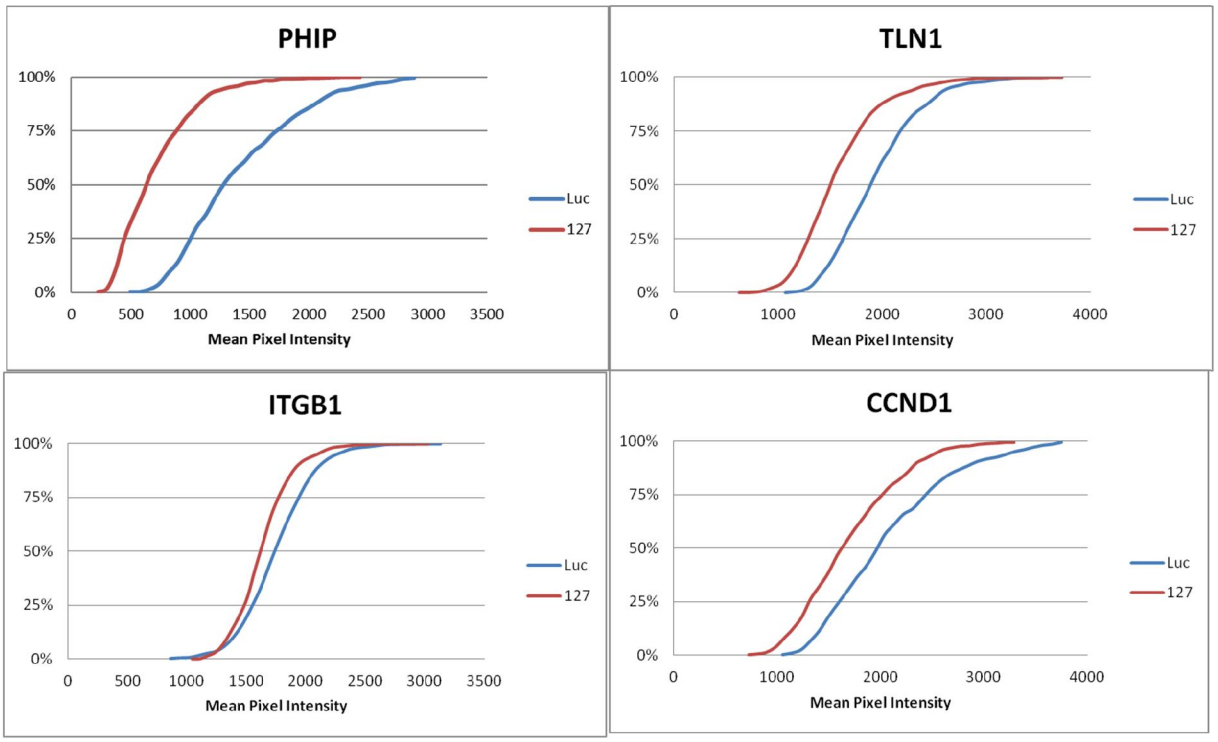
| **Protein tested for** | **H1703 luc** | **H1703 127** |
|---|---|---|
| Merge |  |  |
| PHIP |  |  |
| TALIN 1 |  |  |
| DAPI |  |  |

**Table 7**: Mean Pixel intensity for every protein tested in H1703 via IF




Tables 4 to 6 are showing the result of the IF performed on the H1703 cells. The number of cells differs between H1703 luc and H1703 127 cells as they were not regularly counted before plating the cells on the coverslips. Also during the procedure of the IF cells are washed away on a regular basis, so the number of the cells can't be controlled. For the evaluation of the IF this fact is also irrelevant as the only important factor is the intensity of the different proteins indicating the level of expression.

Figure 6 is showing the correspondent graphs obtained by analyzing the pixel intensity of a selected range. It is important that the area consisting of cells in this range is the same in the control and PHIP knockdown cells to get reliable results.

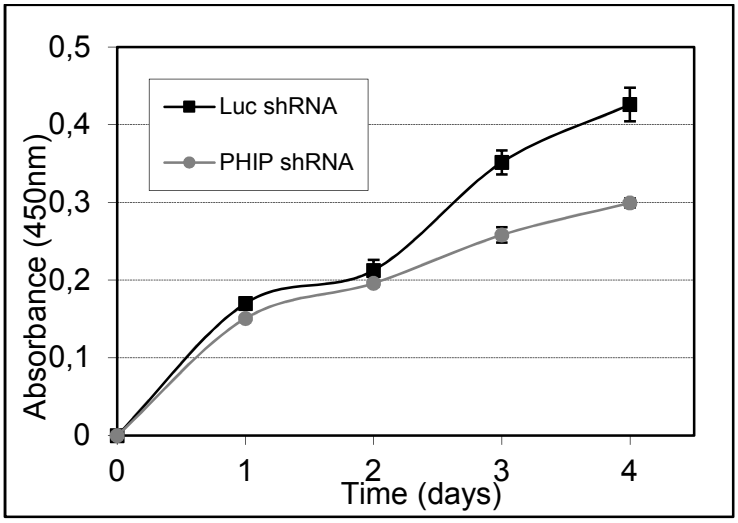
As can be seen PHIP is clearly down in H1703 127 in the IF images, confirmed by the data in the graphs. The more to the left the graph is the lower the pixel intensity and therefore the lower the protein concentration. The y-axis is indicating the percentage of pixels in the selected area having the specific pixel intensity. There is also a significant reduction of TLN1 and CCND1 visible in the PHIP knockdown cells. A small reduction of

ITGB1 levels in H1703 127 cells compared to H1703 luc cells can be seen in the graph and also in the corresponding image, showing that the intensity of the green color is not that much reduced as in the other results. TLN1 is a cytoskeleton protein and plays a role in the function and assembly of actin filaments and in migration of cells. TLN1 also assembles with ITGB1 aiding in cell-cell and cell-surface attachments. Integrins link the cell via the actin cytoskeleton to the extracellular matrix but also play a role in embryogenesis, metastasis of tumors and is also bound to antigen receptor. CDK1 forms a complex with CDK4/6 which regulates G1 to S transition in the cell cycle.
Also it can be seen that PHIP can not only be found in the cytoplasm but also in the nucleus, this is especially true in picture 4.
When combining the stainings of PHIP and the other protein tested for as well as the DAPI-staining a combined picture is the result. This indicates whether PHIP is binding to the other proteins or not.

### 3.4. Cell survival assay/Growth curve analysis

The first assay that was conducted was the cell survival assay. For this 5000 cells are plated in each well and over a course of days the absorbance is measured. This is done by treating the cells every 24h before measuring them with the Dojindo reagent in order to measure their absorbance at 450nm in a spectrophotometer.

**Figure 6**: Comparison of growth curves for H1703 luc and H1703 PHIP

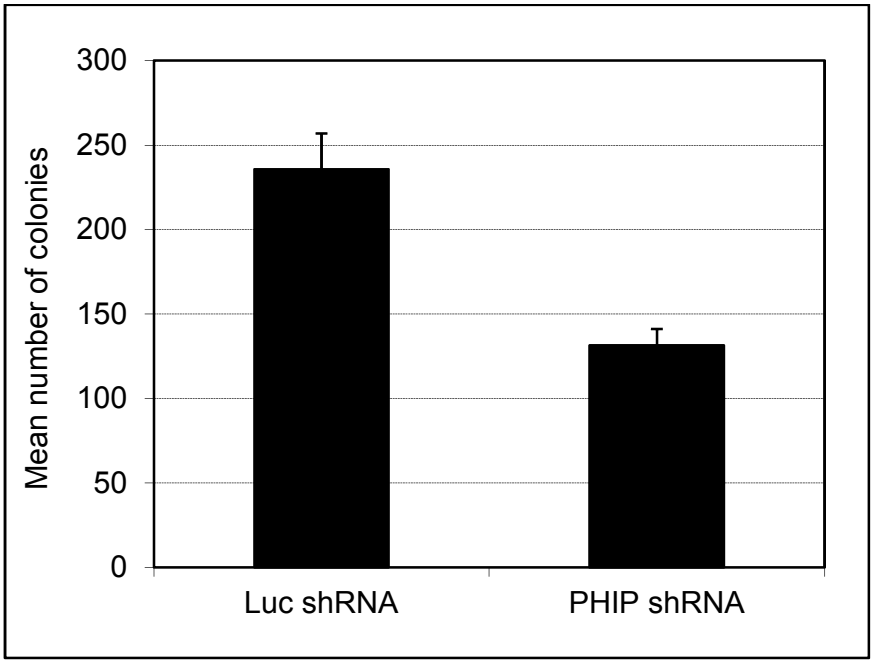


One can see that the control knockdown cell line had more growth than the PHIP knockdown cell line, especially visible after more than 2 days.

### 3.5. Colony formation

For performing a Colony formation assay 2000 cells were plated for both knockdown cell lines and after a few days in culture without any passaging or media change they will start to form colonies. After 9 days visible colonies were seen with the bare eye and were stained, counted and quantified using the available software ImajeJ computer program.

**Figure 7**: Colony formation assay showing H1703 luc and H1703 PHIP

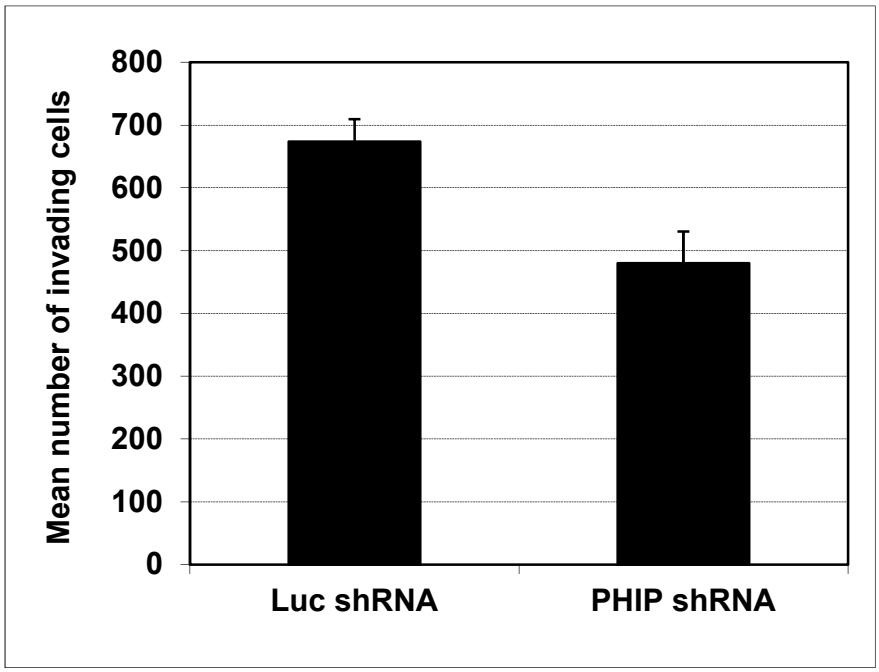



Graph 7 is showing the number of colonies for the respective knockdown cell line in H1703 cells, with the luc knockdown having nearly double the amount of colonies compared to the PHIP knockdown (235 for H1703 versus 131 for H1703 PHIP).

### 3.6. Invasion assay

For this assay 150,000 cells are plated in a matrigel-coated chamber containing media with very low FBS, the well the chamber is placed in contains high-serum media, so the cells are trying to migrate through the matrigel and through the chamber in order to reach the high-serum media. The cells that migrated through are then stained and counted. The density of the Matrigel used for this assay was 5mg/ml and 15µl Matrigel were evenly spread in each chamber.

**Figure 8**: Invasion assay performed on H1703 luc and H1703 PHIP. 1500 cells were plated onto 15µl solidified 5mg/ml Matrigel. Gradient used: FBS gradient

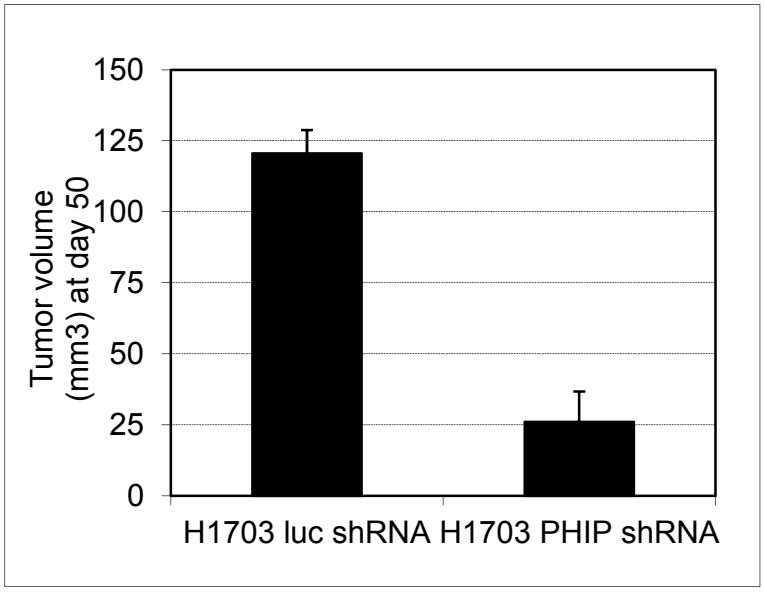



The mean number of invaded H1703luc cells is around 673 cells per plate and the mean number of H1703 PHIP cells that were able to migrate through the Matrigel is 480 cells per plate. This means there is around 29% less invasion in the H1703 PHIP cells found.

### 3.7. In vivo Tumor volume experiment

For this experiment 5 million H1703 luc or 127 cells(in a matrigel/media mix) were injected into 10 or 6 mice respectively and the resulting tumor volume measured at multiple time points. For simplicity reasons only the measurement on day 50 and the last measurement on day 60 is shown. The reason for only using 6 mice in the H1703 127 group was because there were not enough cells available at that time point.

**Figure 9**: Subcutaneous tumor volume ($mm^3$) ofH1703 luc versus H1703 127 stable cells injected in mice




For the first measurement at day 50 after injection the difference between the two groups can be seen clearly. The mice that got injected with the PHIP knockdown cells showed much smaller tumors in general than the group that got injected with the control cell line.

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For the statistical analysis the tumor were ordered according to their size in the beginning, so smallest tumor in group A is grouped together with smallest tumor in group B. When a mouse in one group dies or has to be killed also the correlating tumor in the other group is excluded from the statistical analysis. With this the examiner avoids

to change the outcome of the experiment favoring the desired or projected result. For example when a mouse in group A (injected with H1703 luc e.g.) dies and was having a rather big tumor in the beginning it would be favorable for the experiment if for this mouse a mouse having a small tumor would be left out of the consideration in group B. This is avoided by grouping the mice according to their tumor size.

# 4. Discussion

## 4.1. Generation of knockdown cell lines

As the generation of knockdown cell lines was the first step and necessary to carry out all the other experiments it is crucial in order to be able to continue with the project. As together with the sh-RNA targeting PHIP and luciferase also a puromycin resistance gene was cloned into the vector and transferred to the cells we were able to eliminate cells that did not carry the vector in them by culturing the cells in media containing puromycin as explained before. The successful knockdown of the PHIP gene and in the next step also the PHIP protein was proven via the taqman assay and western blotting. As we only did perform a knockdown and not a complete knockout of the PHIP gene we were especially interested in the levels of the PHIP mRNA and protein in the cells.

## 4.2. Taqman

Figure 4 shows that H1703 shows a 84% reduction in PHIP mRNA levels when compared to H1703 luc whose PHIP levels were assumed to be 100% as a knockdown of luciferase is not having any effect on the other proteins. A knockdown level of 84% is quite high and therefore we decided to continue the experiment with this cell line. A standard deviation of 0.066 for three measurements of H1703 127 tested for PHIP and a standard deviation of 0.056 when testing them for UBC, the control gene is low and therefore again proving that the high knockdown level is true and can be trusted.

### 4.3. Western Blot:

As low mRNA levels do not necessarily mean low protein levels (eg. degradation of mRNA after translation but high half-life of protein) the protein level has to be checked after successful mRNA knockdown. In this case it can clearly be seen that also the PHIP protein was successfully downregulated. AKT and GAPDH levels should not be affected by the PHIP knockdown which was also proven, so this results makes sure that the same amount of protein level was loaded into the gel and that the PHIP knockdown is not a result of differences in protein loading.

Interestingly pAKT, the active protein in the cell, is reduced in H1703 127 cells, although the inactivated AKT level remains constant. This means that knockdown of PHIP has a direct or indirect effect on the phosphorylation status of AKT and is a strong indicator that PHIP is exerting its effects over the AKT-pathway.

### 4.4. Immunofluorescence staining

Immunofluorescence staining is another way to visually show protein knockdown and localization in the cell. Also the images can be quantified to have a scientific result showing protein levels.

For the IF-staining the cells are always stained with two different AB against PHIP and another protein to be investigated. Also the nucleus is stained and appears blue. The IF results show that with the knockdown of PHIP also TLN1, ITGB1 and CCND1 are downregulated, although the difference in ITGB1 is not that significant. This means that PHIP is also having an effect on these proteins.

As described above these 3 proteins do have an important role in cancer progression and in the regulation of different factors important in the formation and development of cancer cells.

As seen in Table 7 knockdown of PHIP is also inducing a slightly downregulated level of these 3 proteins in the cells, again either through a direct or indirect effect.

Only after the positive completion of these experiments, proving the significant knockdown of PHIP we were able to conduct the assays in order to answer the research question how the downregulation of PHIP is changing cell behavior. Therefore a cell survival, colony formation and invasion assay was carried out.

### 4.5. Cell survival

Usually cell survival is used for drug testing but in our case we were not interested in the effect of a drug on the cells but rather on the effect of PHIP knockdown on the cells. As it can be seen in Figure 6 the H1703 127 show reduced growth, meaning that a PHIP knockdown decreases the growth and ability to sustain and adapt to a changing environment that is not providing optimal growth factors. In turn this means that high PHIP levels improve growth and survival in different conditions.

### 4.6. Colony formation assay:

Another assay assessing the growth and survival of cells is the colony formation assay. Graph 7 is showing a decreased number of counted colonies in the PHIP knockdown in the PHIP knockdown cells. The colony formation assay is used to determine the ability of one single cell to build or grow into a colony, consisting of at least 50 cells. This again is showing the ability of unlimited division, so high PHIP levels mean a high ability to avoid death and reproduce in a fast and efficient manner.

### 4.7. Invasion assay

As the name already suggests the invasion assay determines the invasive ability of the cells tested and as it can be seen in Figure 8 a PHIP knockdown is significantly reducing the invasive ability of cells. This can be an important finding when looking into in vivo models as the invasive ability of cells is directly linked with metastasis as the cells first need to invade through ECM and tissue surrounding the tumor before they can metastasize in different parts of the body.

### 4.8. In vivo tumor growth

The in vivo tumor growth experiment is the last experiment performed and gives the ultimate result and hint for PHIPs influence on cell growth and tumor formation not only in vitro but also in vivo. A knockdown of PHIP is reducing the tumor volume significantly as it can be seen in Figure X. Interestingly the differences between the two cell lines did not remain that big and constant throughout the experiment. The second graph shows that the tumor volume of the H1703 127 injected cells did increase significantly over a

time course of 10 days. The most obvious and prominent reason for this is the loss of the shRNA with increasing tumor size, as the cells were not under puromycin anymore.
Another reason for this phenomenon is the ability of different mice to suppress or cope with the tumor. Every animal has a different health status, even though they were treated the same way and therefore tumors even regressed in some of these mice after an initial growth. If this did happen or if a mouse died it was considered during the statistical analysis as mentioned above. Furthermore in vivo processes like vascularization or ulceration play a role in the growth and health status of the animal.

### 4.9. Summary and outlook

To summarize reducing the expression of PHIP by targeting it with a specific shRNAsignificantly affected proliferation and invasive ability of the cells as well as tumor growth in immunodeficient mice. Furthermore it is having an effect on the levels of other proteins in the cell known to play a role in cancer progression such as AKT, ITBG1, TALIN 1 and CYCLIN D1.
This is suggesting PHIP not only as a marker for progression in lung cancer but also as a rational target for lung cancer treatment.
The next step in achieving this goal would be to acquire a deeper knowledge in the exact pathways leading to the effects of high PHIP levels on cancer cells and tumors in general. Also the establishment of a screening procedure for PHIP levels on a regular basis not only in melanoma or lung cancer patients but also in patients suffering from other cancer types is carried out and will also continue in the future.
Another aspect is to find a drug that targets PHIP in vivo with bearable toxicity and side effects, one approach for this is the Bromodomain inhibitor JQ1 that is a prominent and known drug for targeting bromodomains in many proteins and was found to target PHIP in an acceptable manner (results not shown). The goal is to find a structure by chemical analysis specifically targeting PHIP with considerable high tolerance in patients as an additional therapy accompanying surgery and/or chemotherapy.

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