4 Results

4.1 shRNA induced prohibitin knockdown

To induce a stable repression of prohibitin protein levels, shRNAs targeting either the coding region or the 3'UTR of the prohibitin mRNA were cloned into the pLL3.7 lentiviral vector system. The expression of the shRNA was therefore under the control of the polymerase III promoter U6 and linked to the expression of the marker protein GFP.

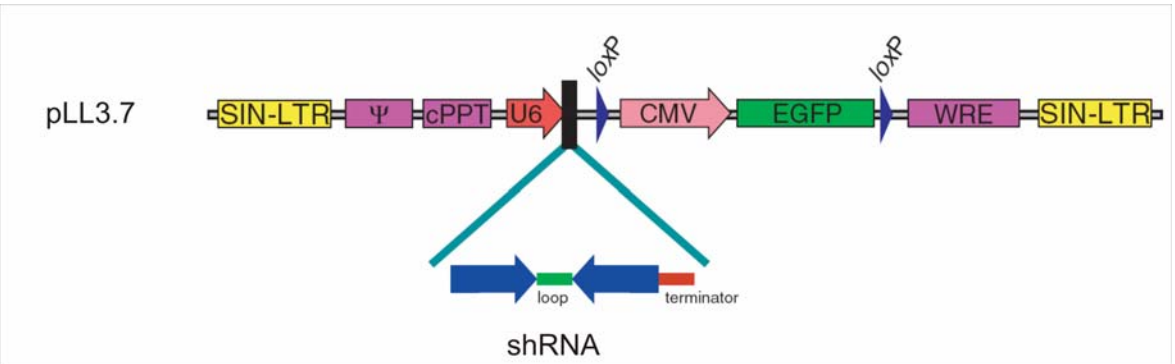

Figure 4-1: Prohibitin shRNAs and control shRNAs were cloned into the pLL3.7 vector system.
To generate a constitutively active knockdown of Prohibitin, a set of different shRNA cassettes was cloned into the lentiviral shRNA expression vector pLL3.7 under the control of the polymerase III promoter U6. Belonging to the 3rd generation of lentiviral vectors, generation of lentiviral particles was done by transfecting 293T cells with the corresponding packaging plasmid (see Chapter 7.1.3.1). Cells were transduced with an MOI of 5 and 10µg/ml polybrene in medium. Infection efficiency was tracked by assessing GFP expression using FACS analysis.

After successful cloning of the shRNA cassettes, viruses were produced, which were then used to transduce HeLa cells. Knockdown efficiency of the shRNAs was validated, at both the RNA and protein levels by obtaining lysates for qRT-PCR and western blot analysis (Figure 4-2).

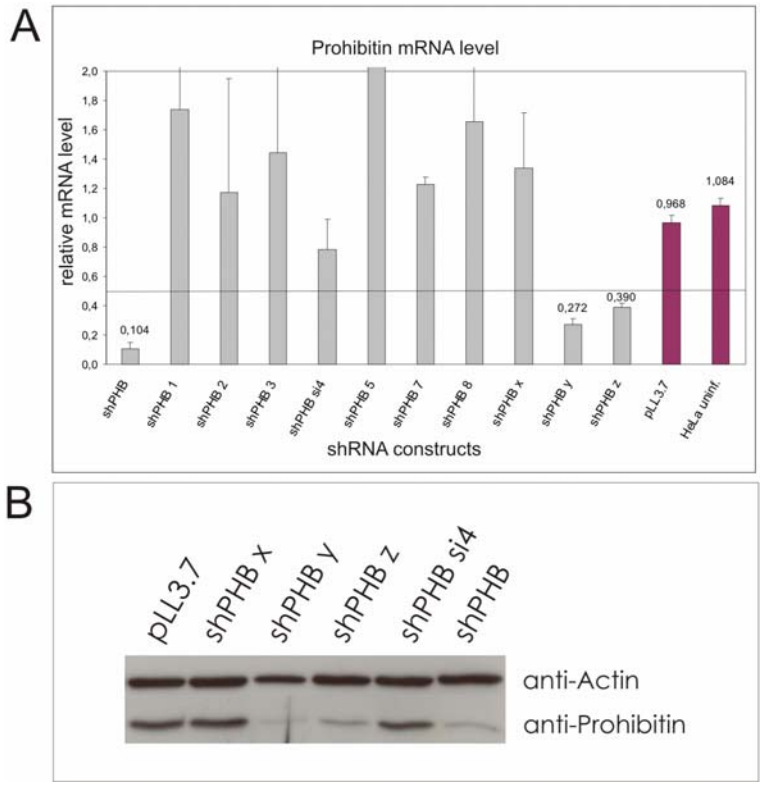

Figure 4-2: Expression of the shRNAs shPHB, shPHBy and shPHBz
The expression results in knockdown of prohibitin mRNA and protein. HeLa cells were transduced with lentiviral particles expressing different prohibitin targeting shRNA or the control vector (pLL3.7) **A**, Five days post infection cells were lyzed and the prohibitin mRNA level was measure by rtPCR with reference to GAPDH mRNA expression. **B**, The protein level in transduced cells was assessed by western blot. Protein levels were normalized using the signal obtained for actin.

Of the 14 tested shRNAs only three resulted in a knockdown within the definition of 60%-70% RNA reduction. These also exhibited a strong knockdown at the protein level and were therefore used to generate stable prohibitin knockdown cell lines.

4.2 Prohibitin knockdown in cancer cells

Prohibitin 1 has been previously ascribed a role as a tumor suppressor[52; 87; 88], however this view has often been challenged[12; 76; 76; 77]. Previous experiments using siRNA transfection to transiently knockdown prohibitin 1, demonstrated its role as a protein necessary for cell survival[69]. It was also suggested that prohibitin 1 is necessary for the activation of C-Raf, which is a protein involved in growth factor signaling and is frequently mutated into a permanently active form in tumor cells. This hypothesis was supported by the demonstration of direct binding of prohibitin and C-Raf[89].

To further evaluate the role of prohibitin 1 in carcinogenesis a set of different cancer cells was transduced with lentiviral particles to express prohibitin specific shRNAs or control shRNAs, respectively. Hence, the positively validated shRNAs, shPHB, shPHBy and shPHBz as well as a control shRNA targeting luciferase (shLuci) or empty vector (pLL3.7) were used in all of the following experiments. The cell lines chosen for analysis of a prohibitin knockdown represent a cross section of cancer cells with mutations in Ras and/or Raf proteins.

cell line	**cell type**	**mutation**
IF6	Melanoma	B-raf
HT29	Colon, colorectal adenocarcinoma	K-/H-/N-ras, B-raf
CapanI	Pancreas, adenocarcinoma	K-ras
HT1080wt	fibrosarcoma	N-ras

Table 1: Cancer cells expressing mutations in Ras and/or Raf proteins

As GFP expression is the marker for successful transduction, the infection rate was gauged by its fluorescence intensity in FACS analysis. Earlier experiments demonstrated that GFP expression correlated with a prohibitin protein knockdown (data not shown). The GFP expression 3 days post infection was close to 100% for shRNA expressing cells and the empty vector control expressing cells, indicating a high infection rate. However, within one week of infection, the GFP expression was reduced in prohibitin knockdown cells only and almost lost within three weeks post infection (Figure 4-3). This was observed in all infected cell lines including Jurkat cells and HUVEC cells and therefore not limited to epithelial cells or cancer cells. Although this was an initial observation, it clearly showed that prohibitin protein expression was necessary for maintaining the homeostasis of all tested cells.

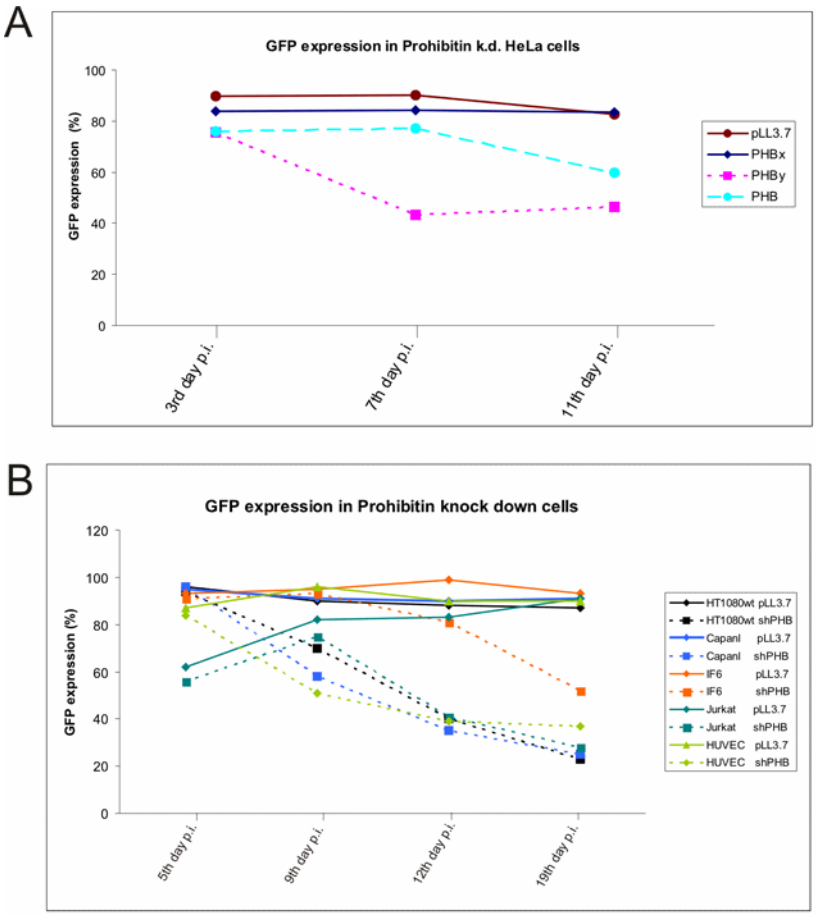

Figure 4-3: Prohibitin knockdown leads to reduced GFP expression in different cancer cells
GFP is the marker protein for infection and shRNA expression. At different intervals post infection (*p.i.*) with prohibitin shRNA and control vector (pLL3.7) carrying lentiviral particles, GFP expression was gauged by FACS analysis and the percentage of GFP expressing cells was assessed. **A**, The proportion of GFP and prohibitin shRNA expressing cells was reduced in HeLa cells within 7 days *p.i.* **B**, GFP expression was lost in different cancer cells and primary cells within 9 days of infection with shPHB carrying lentiviral particles. *Solid lines*, control vector group values. *Dashed lines*, prohibitin knockdown group values.

The loss of prohibitin and, consequently, the loss of cancer cells suggested an inconsistency in at least one of the six hallmarks of cancer, defined by Hanahan and Weinberg[31]. The loss of GFP expressing cells, equatable to cancer cells exhibiting a prohibitin knockdown, may have occurred in a number of ways. As reduced GFP expression could not have resulted from spontaneous apoptosis, because an increase in cell blebbing or the formation of apoptotic bodies was not observed, the proliferation efficiency of HeLa cells devoid of prohibitin was analyzed. This approach assumed that the proportional loss of GFP expressing cells was the result of overgrowth by non infected cells which did not show a knockdown in prohibitin expression.

4.3 Proliferation in prohibitin knockdown cells

To obtain quantitative information of the proliferation rate of HeLa cells with a prohibitin knockdown, cells were seeded in the presence of BrdU, a thymidin analogue, the incorporation of which was quantified by immunodetection via a peroxidase conjugated antibody.

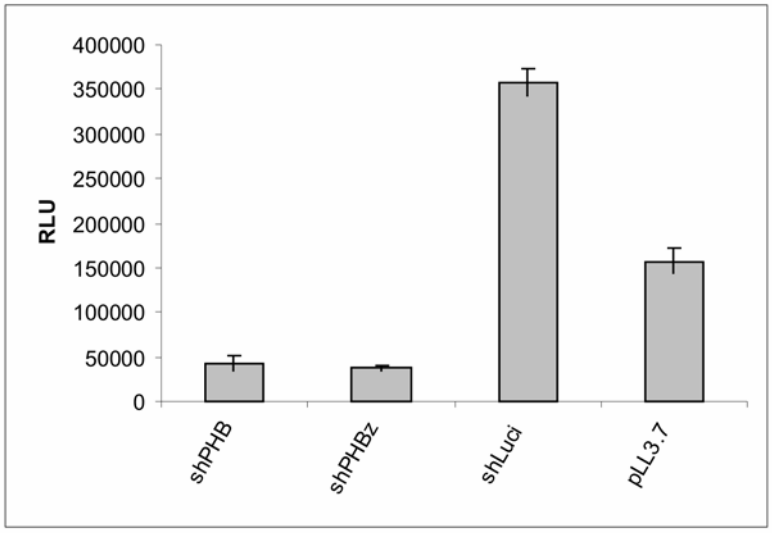

Figure 4-4: Reduced BrdU uptake in HeLa cells expressing prohibitin shRNAs
To measure DNA synthesis prohibitin knockdown and control cells were seeded 24h before analysis. 1h before fixation, 10µM BrdU was added. BrdU incorporation was visualized by chemiluminescence using anti-BrdU-Peroxidase and the relative luminescence was assessed.

Prohibitin knockdown cells showed significantly less BrdU incorporation (Figure 4-4). As BrdU is incorporated in the S-phase, a block in BrdU uptake is generally associated with a block in G_1/S-progression[22]. However, the BrdU incorporation was measured via intensity per well and not on single cell level, therefore the reduced incorporation of BrdU could also be explained by a decrease of the cell number per well. This made this assay useful to quantify the proliferation defect in prohibitin knockdown cells, but not to assess a potential block at a certain cell cycle checkpoint.

The ratio of the different cell cyle phases was measured by quantitative propidium iodide staining of cellular DNA (Figure 4-5). Stoichiometric intercalation between the DNA bases allowed a distinction between single stranded DNA in the G_1 phase or double stranded DNA in the G_2/M phase, with the S-phase found between these peaks. The analysis of the different states of the cell cycle did not reveal a block in the G_1/S-phase transition nor at another cell cycle checkpoint.

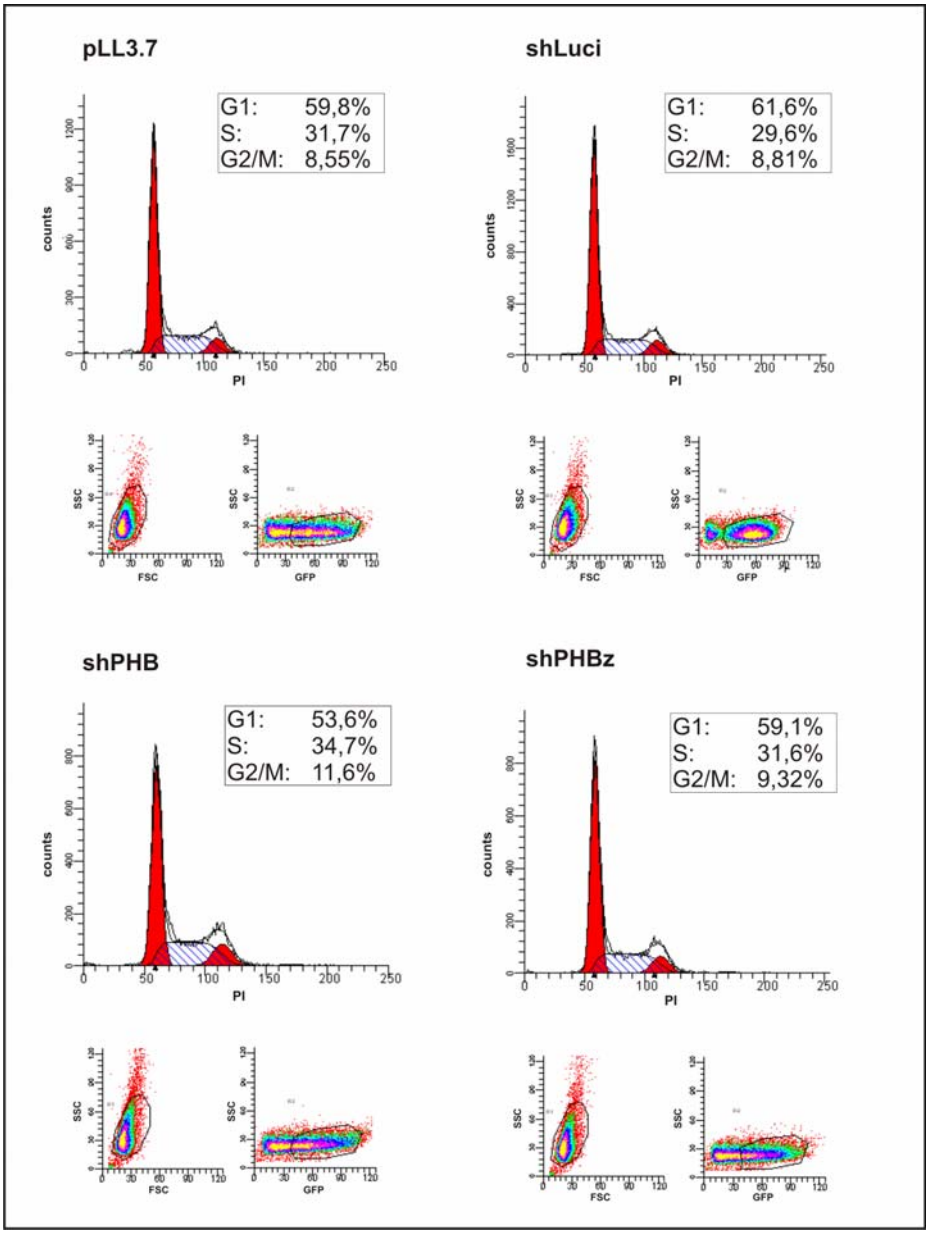

Figure 4-5: DNA staining with propidium iodide did not reveal a block in cell cycle progression.
Five days post infection, cells were fixed in 4% PFA and DNA was stained with propidium iodide (PI) to evaluate cell cycle distribution by FACS analysis. The total cell population was gated on GFP positive cells and the number of PI positive cells in the respective cell cycle stadium was assessed by means of fluorescence intensitiy using the deconvolution software 'ModFitLT'. The rate of cell debris and aggregates was less than 3% in all samples. This experiment was done by Gwendolyn Billig.

Although a drop off in prohibitin knockdown cells was reproduced consistently, a block in cell cycle progression was not identified. Reduced prohibitin expression led rather to a slackened turnover in cell cycle transition. Furthermore, analysis of prohibitin knockdown cells towards an arrest in G_0-phase, a condition in which senescence associated (SA)-β-Galactosidase was expressed and measured[35], did not reveal a disruption of a limitless replicative potential (data not shown).

4.4 Ras-Raf-MEK signaling in prohibitin knockdown cells

The above and previous experiments[69] led to the hypothesis that a block or reduction in growth factor signaling might be involved in the reduced proliferation of prohibitin knockdown cells. There was convincing evidence that particularly a block in the Ras/Raf/MEK-signaling pathway was the cause of the observed proliferation defect, as the role of prohibitin as a directly interacting partner of C-Raf was confirmed (Figure 4-6).

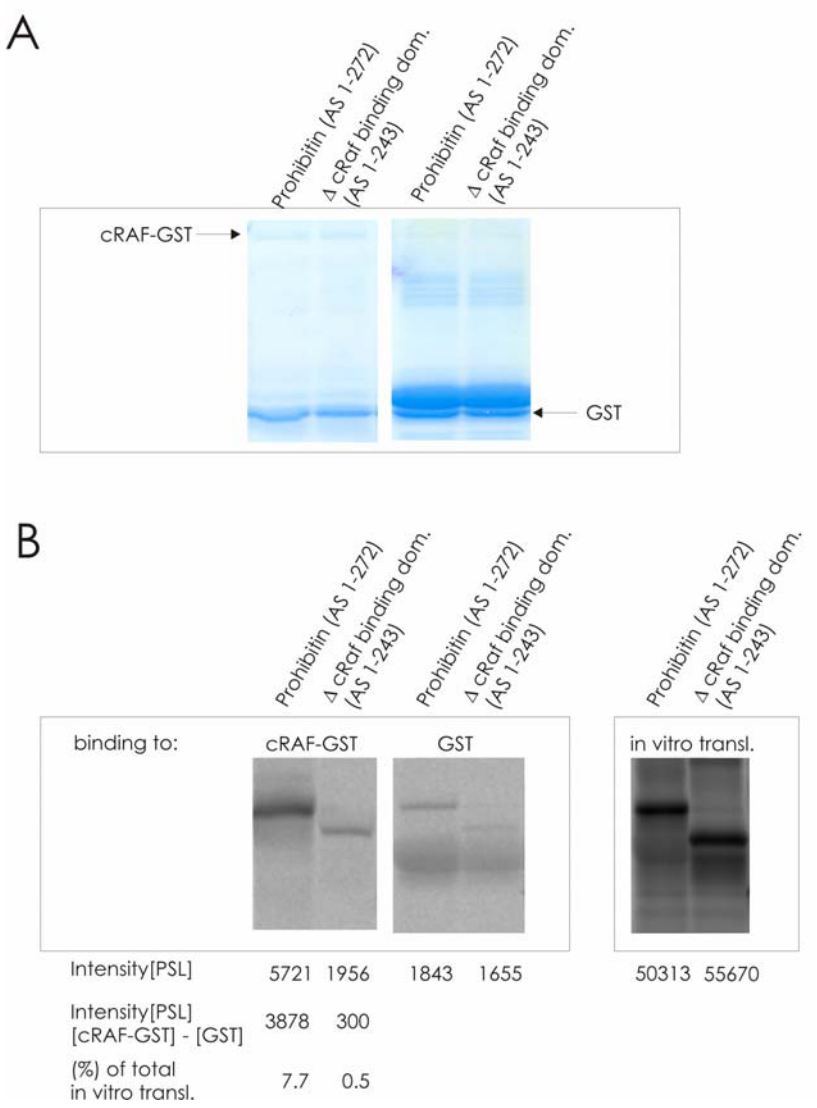

Figure 4-6: Prohibitin interacts with C-Raf via its c-terminal Raf binding domain.
C-Raf-GST was overexpressed and purified from SF9 cells. Prohibitin-his tagged proteins were translated *in vitro* in reticulocyte lysates in the presence of ^{35}S-Methionine. After the binding of prohibitin proteins to GST-trapped C-Raf-GST for 2h at 4°C, soluble complexes were eluted with 20mM glutathione and protein content was assessed by **A**, coomassie gel and **B**, auto radiography.

For that purpose C-Raf-GST fusion protein was purified from SF9 cells, a gift from the Rapp laboratory, Würzburg, and incubated with *in vitro* translated prohibitin-His

tagged proteins, both the full length version and the C-terminal deletion construct AS 1-243, respectively. Only full length prohibitin could effectively bind to C-Raf, while deletion of the C-terminus abrogated the binding to C-Raf.

The Ras/Raf/MEK signaling pathway, which can be activated through the binding of a ligand to EGFR, was therefore analyzed more closely. Under serum starved conditions, the addition of EGF led to the activation of Erk by phosphorylation. In the case of a block in EGFR signaling, possibly through a block in Raf activation, Erk activation is expected to be reduced. This, however, was not the case in prohibitin knockdown cells. None of the analyzed cell lines showed a reduction in Erk activation as assessed by the detection of phosphorylated Erk in western blot (Figure 4-7).

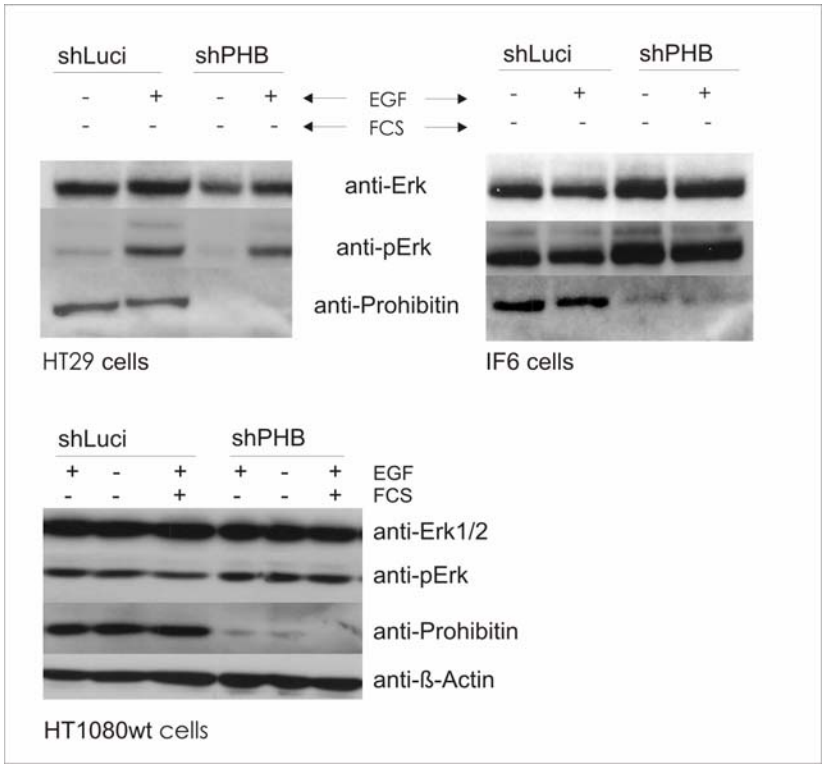

Figure 4-7: The block of proliferation is independent of growth factor signaling.
Different cancer cell lines with mutations in Ras and Raf proteins, expressing a prohibitin shRNA (shPHB) or a control shRNA (shLuci) were analyzed for a block in growth factor receptor signaling. The cells were serum starved for 4h before activation with 25ng/ml EGF for 5 min. Activation was stopped by the addition of 2x sample buffer and Erk activation was assessed by western blot. Protein levels were normalized using the signal obtained with total Erk1/2.

Although it can be assumed that the interaction between C-Raf and prohibitin is abrogated, this loss in interaction did not lead to a deficiency in growth factor signaling. Therefore a defect in the processing of incoming growth factor signals was not inhibited in prohibitin 1 knockdown cells.

4.5 Loss of prohibitin stops anchorage independent growth

Thus far, none of the defined hallmarks of carcinogenesis were found to be abrogated in prohibitin knockdown cells. As cancer cells frequently mutate, obtaining the ability to grow anchorage-independently and therefore acquiring the capacity to metastasize, a block in this transformation process was analyzed. To this end, prohibitin knockdown cells were seeded at low density under single cell conditions in soft agar (Figure 4-8). Whereas control cells, infected with empty vector virus (pLL3.7) were still able to grow under these anchorage-independent conditions, cells expressing a prohibitin shRNA (shPHB, shPHBz) stopped growing and did not form colonies. This experiment showed that the reduced prohibitin expression resulted in the loss of transformation. All tested cancer cell lines were not longer able to grow anchorage independent.

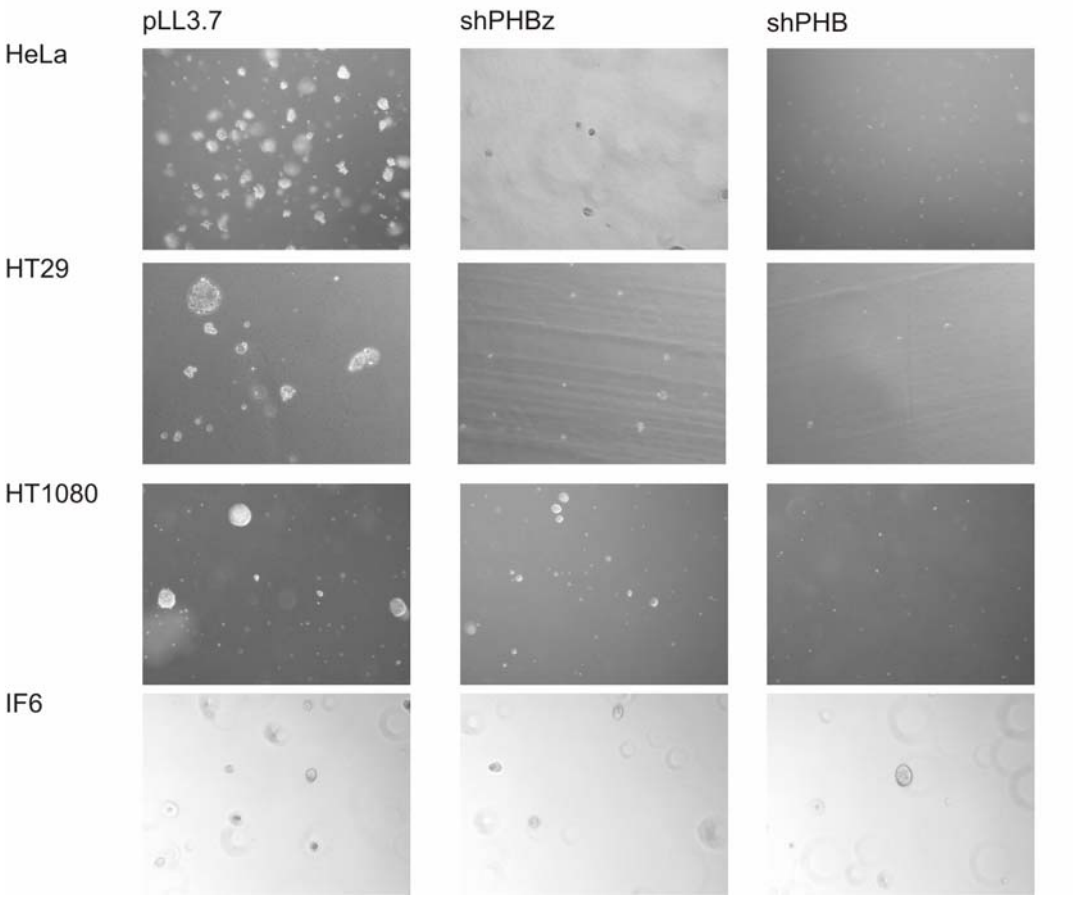

Figure 4-8: Loss of prohibitin stops colony growth in soft agar
Different cancer cell lines infected with prohibitin shRNA and control lentiviral particles were seeded at a density of 500-2000 cells/ml in 0.3% low melting point agarose in cell culture medium. Colony formation was observed within a timeframe of 2-6 weeks.

4.6 Doxycycline inducible prohibitin knockdown

Until now, experiments were performed on a pool of transduced cells. As prohibitin knockdown cells were lost from the pool within one to two weeks, the time frame for experiments was limited to five to six days, making it impossible to carry out experiments that required a large amount of cells. The pLVPT one vector doxycycline inducible system (provided by D.Trono) was therefore established[81]. This vector transcribes the shRNA under the control of the polymerase III promoter H1 and expresses GFP as a marker protein, transcribed from the PGK promoter. The function of both promoters is blocked through binding of the fusion protein (between KRAB and the tet Repressor (tetR)) to the tet Operon which is positioned in front of the H1 promoter. The repressor is also transcribed from the PGK promoter and is positioned behind an IRES site.

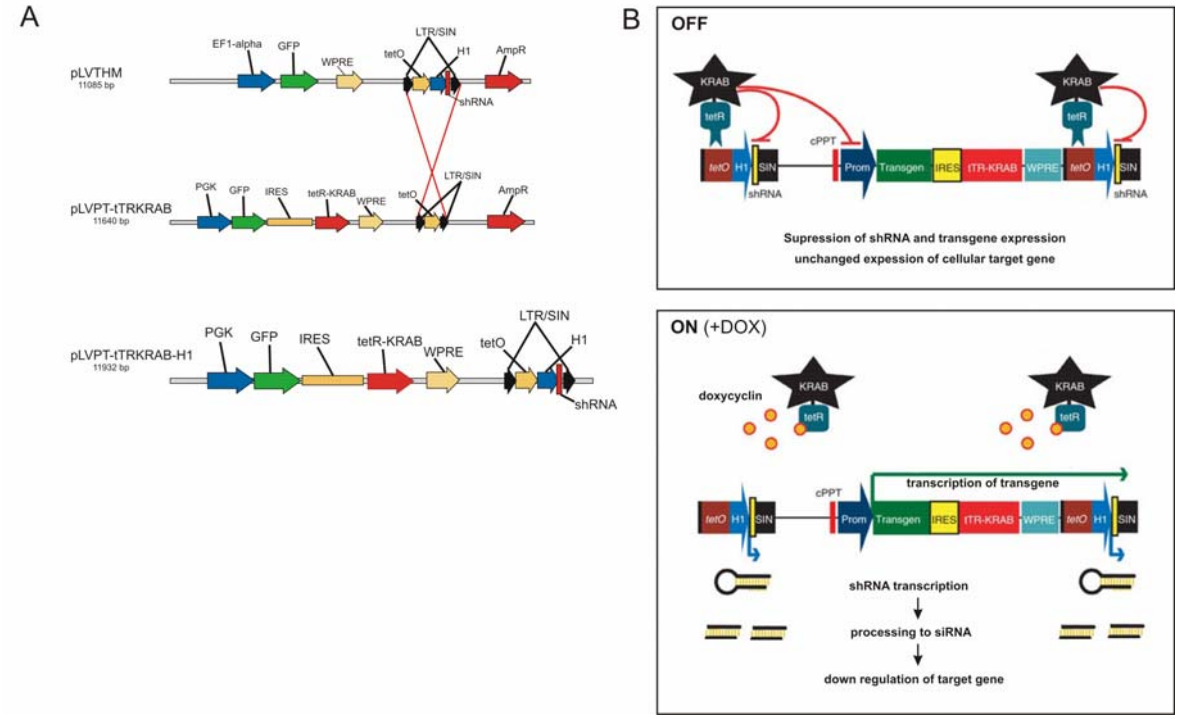

Figure 4-9: The pLVPT one vector, doxycycline inducible lentiviral vector system.
A, To generate an inducible knockdown of prohibitin, the shRNA cassettes shPHB, shPHBz and shLuci were transferred from the pLVTHM lentiviral vector containing the H1 promoter to the pLVPT vector (both of which were generously provided by D.Trono, Geneva), causing in the latter the shRNA expression to be controlled by a doxycycline regulated polymerase III promoter. Viral particles were generated using the packaging plasmids of the 2nd generation of lentiviral systems (see Chapter 7.1.3). **B**, Expression of shRNA was induced by adding 1µg/ml doxycycline to infected cells. Infection efficiency was tracked by assessing GFP expression using FACS analysis.

Under non-doxycyline conditions enough repressor was made to block the expression of shRNA and marker protein. The addition of doxycycline (1µg/ml) led

to the release of the repressor from the tet Operon and consequently to the transcription of shRNA and GFP (Figure 4-9B).

In order to insert the shRNA into the pLVPT vector, the shRNA cassette was first cloned into the pLVTHM vector under the control of the H1 promoter, then exchanged from the pLVPT vector via homologous recombination with the LTR tet operon cassette (Figure 4-9A). These pLVPT constructs were used for the generation of lentiviral particles. The transduction efficiency was defined by assessing GFP expression using FACS analysis. After administration of 1µg/ml doxycycline, prohibitin protein expression decreased in the cells within four days of induction and expression was stably suppressed for >14 days (Figure 4-10A).

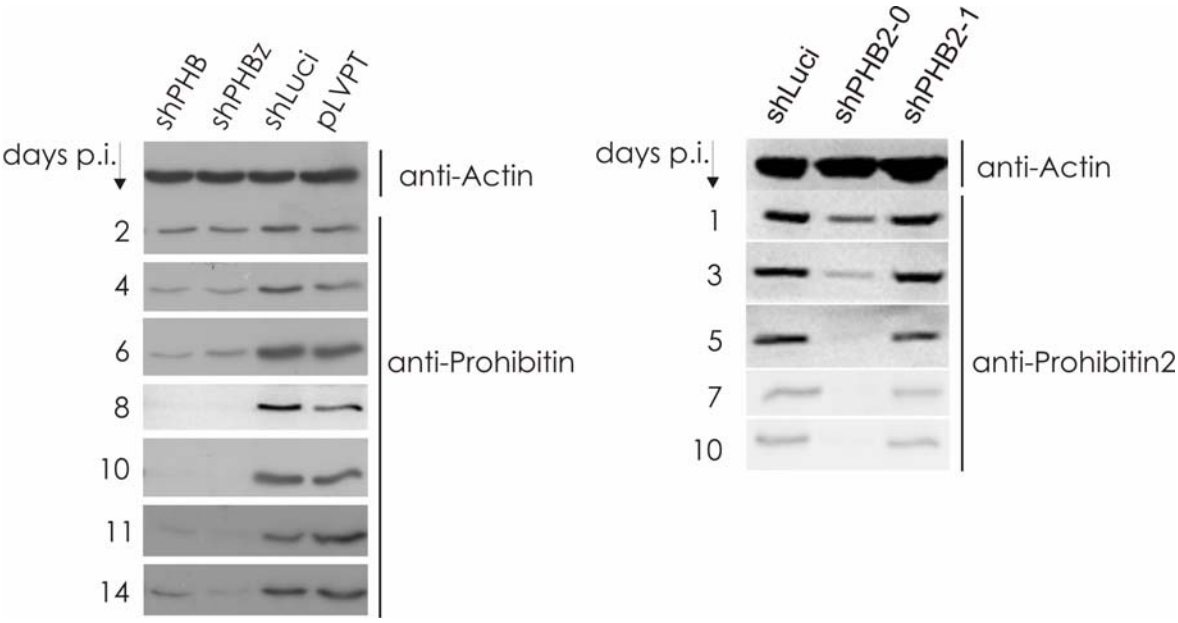

Figure 4-10: Transduced HeLa cells show a doxycycline inducible knockdown of prohibitin. Infected HeLa cells with >95% GFP expression were induced with 1µg/ml doxycycline for the indicated time and prohibitin protein level was assessed by western blot, using the actin signal to normalize protein level. p.i. – post induction

4.7 Double knockdown: prohibitin 1/prohibitin 2

As it has been reported that prohibitin and its homologue prohibitin 2 stabilize each other on protein level[43], the expression level of both proteins in each knockdown cell line was analyzed. The doxycycline inducible HeLa cell line for prohibitin 2, that expresses the shRNA shPHB2-0, showed a knockdown of this protein within three days of induction (Figure 4-10B).

Prohibitin 1 and 2 mRNA and protein expression levels were analyzed 10 days post-induction with doxycycline. While the mRNA level of one prohibitin was

reduced with expression of the specific shRNA, the level of the other was stable or had even an increase. At the same time prohibitin protein levels were reduced regardless of whether the shRNA expressed had targeted prohibitin 1 or prohibitin 2 (Figure 4-11).

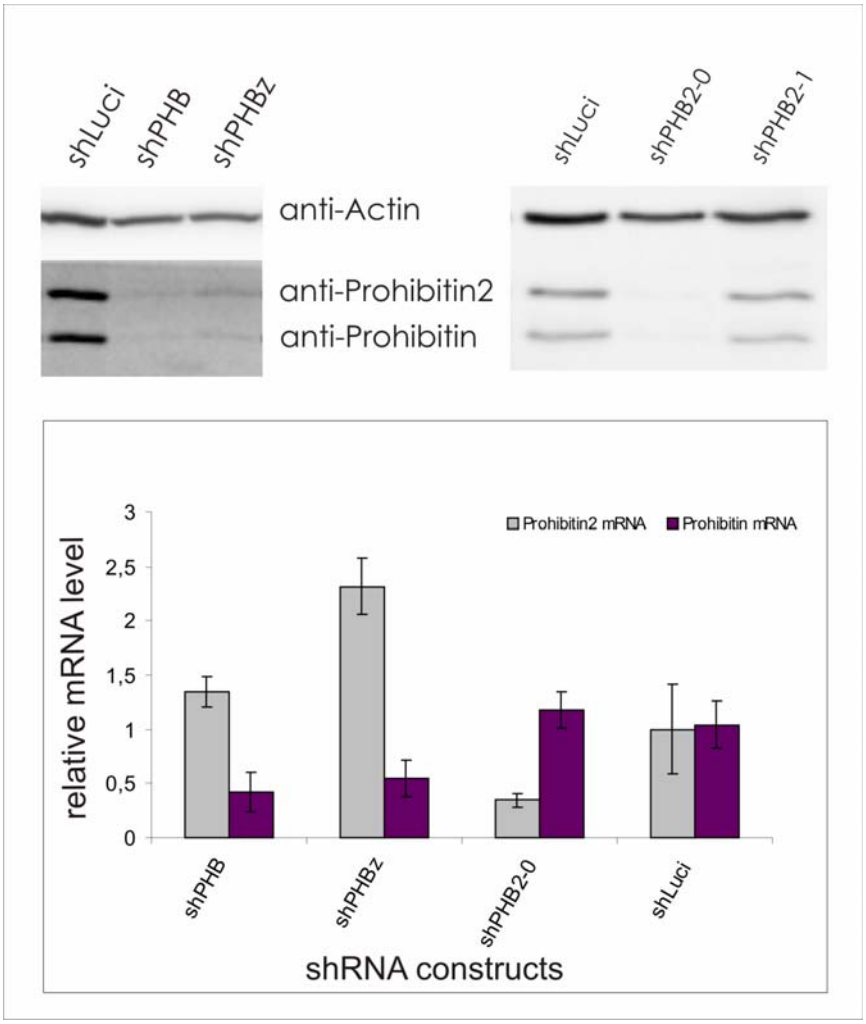

Figure 4-11: Double knockdown of prohibitin proteins through loss of protein stability
A knockdown of one prohibitin protein through specific shRNA targeting led to the loss of the homologous prohibitin protein while the RNA level remained stable.

As both prohibitin proteins were lost upon expression of the shRNAs targeting either of them, it remained unclear whether the loss of prohibitin 2 mRNA by expression of shPHB2-0 resulted in the same proliferation defect. The same phenotype would indicate a regulation at the protein level, while undisrupted proliferation could only be explained by a regulation at the RNA level exclusively for prohibitin 1. Transduced HeLa cells, induced for six days were treated with carboxy fluorescein diacetate succinimidyl ester (CFSE), a fluorescent compound that distributes itself stoichiometricly among the daughter cells, thus halving the

fluorescence intensity with each cell division. Both prohibitin knockdown cell lines showed a reduced proliferation rate as the CFSE fluorescence intensity was reduced more slowly than in control cells (shLuci) (Figure 4-12).

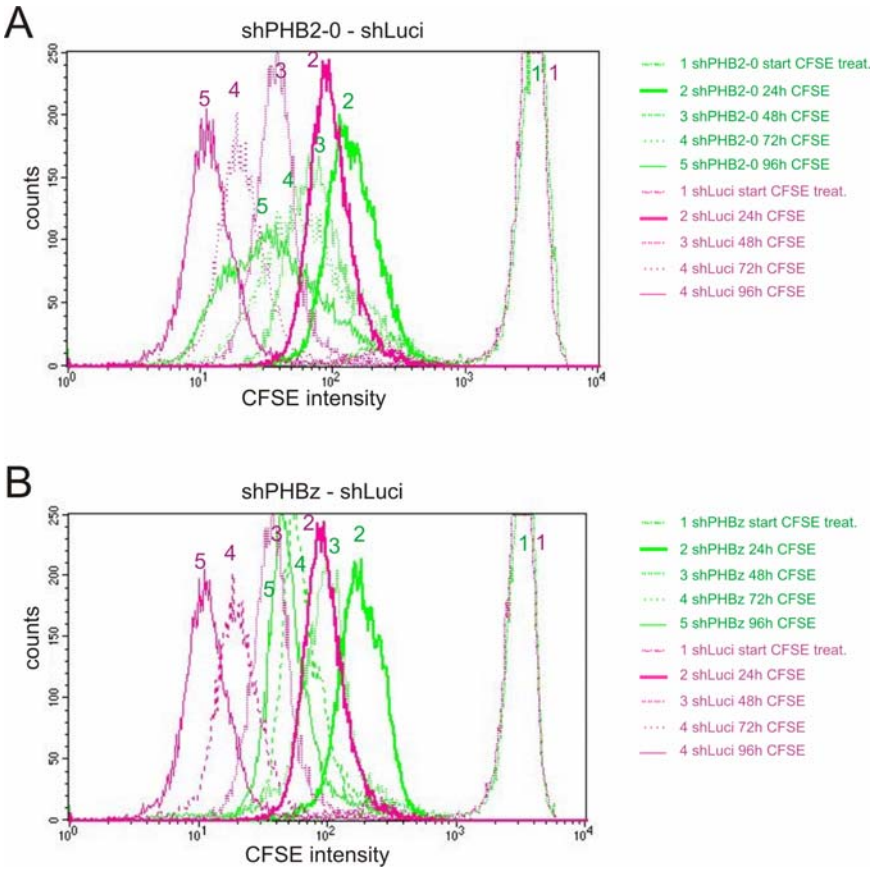

Figure 4-12: Decrease of CFSE intensity is slowed down upon prohibitin knockdown
Prohibitin knockdown was induced with 1µg/ml doxycycline for 6 days before treatment with 5µM CFSE, 5 min at RT. A sample was analyzed by FACS (peak 1). Samples were taken every 24h and CFSE intensity was quantified by FACS. **A**, CFSE intensity of shPHB2-0 cells (green) plotted against shLuci-control cells (pink). **B**, CFSE intensity of shPHBz cells (green) plotted against shLuci-control cells (pink).

4.8 Mitochondrial function of prohibitins

As neither the self sufficiency of growth factors nor loss of limitless replicative potential caused of the observed proliferation defect, further cellular signaling processes were investigated. Both prohibitin proteins are localized mainly in the mitochondria, where they participate in the formation of a complex with the m-AAA protease. This protease is part of the import machinery for integration of proteins from the electron chain complex into the inner mitochondrial membrane. The ATP level in prohibitin knockdown cells was investigated, as malfunction of the m-AAA protease may result in reduced ATP generation in these cells, which in turn could cause the observed reduction in the rate of cell proliferation.

Cells were seeded in microtiter plates and the amount of ATP was measured by utilizing the ATP-dependent conversion of D-luciferin to oxoluciferin, which is catalyzed by luciferase under stoichometric emission of luminescence. The luminescence intensity therefore corresponded to the available ATP level (Figure 4-13). A knockdown of prohibitins did not lead to a decrease in the generation of ATP but rather to an increase in ATP level in shPHBz cells.

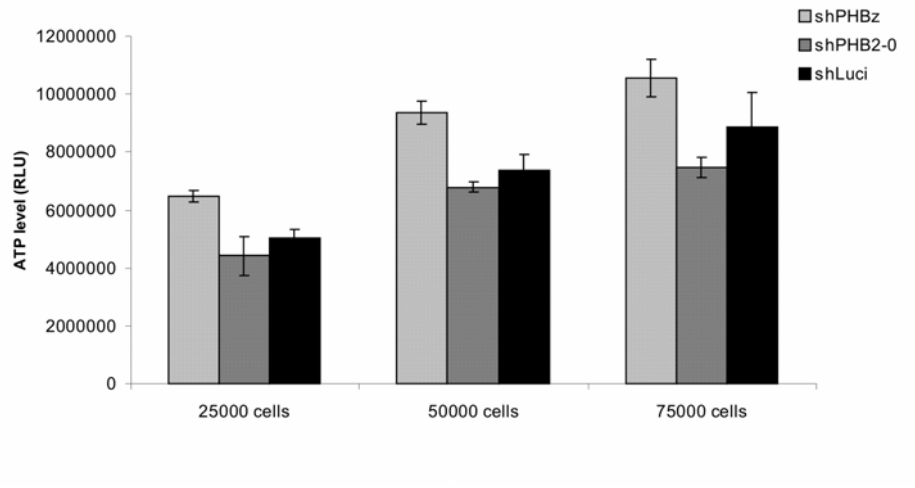

Figure 4-13: ATP levels are not changed in prohibitin knockdown cells.
To measure ATP levels in HeLa cells with a prohibitin knockdown induced for 10 days, the indicated amount of cells was seeded per well on a microtiter plate. ATP levels were assessed by measuring luminescence, emitted in stoichiometric ratio to available and consumed ATP. Prohibitin knockdown cells showed no decrease in ATP synthesis.

As the role of m-AAA protease in the maturation of mitochondrial proteins is not clearly defined, the following experiment assessed mitochondrial protein levels. Mitochondria were purified from HeLa cells with a prohibitin knockdown induced for 10 days and protein levels were determined by western blot. A change in the level of mitochondrial protein expression, triggered by a reduction in prohibitin expression was not detected. Proteins from the mitochondrial import machinery as well as electron chain components and housekeeping proteins were stably expressed (Figure 4-14A). These experiments indicated that reduced prohibitin expression did not lead to a disruption of mitochondria function and thus could have not have been the cause of slowed proliferation.

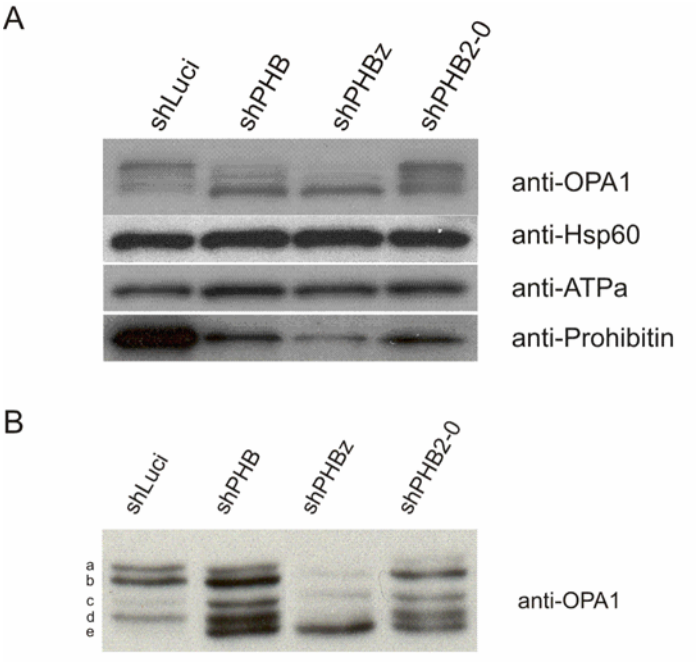

Figure 4-14: A prohibitin knockdown does not lead to a destabilization of proteins from the electron chain complexes but rather to change in the expression pattern of the fusion protein OPA1.
Mitochondria were isolated from HeLa cells expressing prohibitin shRNAs for 10 days and the protein level was assessed by western blot. **A**, Protein expression levels in mitochondria from prohibitin knockdown cells were not changed. Both heat shock protein, 60kDa (Hsp60) levels and ATP-synthase α (ATPa) levels were not changed with reduced prohibitin expression (shPHB, shPHBz and shPHB2-0) **B**, OPA1 protein levels were detected as five isoforms, termed a-e (shLuci). A prohibitin knockdown led to a change in expression levels of these isoforms, as was assessed by western blot. This change was expressed in an increase of isoform e for all prohibitin shRNA expressing cell lines and a decrease for isoforms a, b and d in shPHBz cells.

4.9 OPA 1 fragmentation in prohibitin knockdown cells

The knockdown of prohibitins affected only one of the observed proteins. The expression pattern of OPA1 changed with a nearly complete loss of prohibitins (Figure 4-14B). OPA1 is a mitochondrial protein transcribed from eight splice variants, which again are processed at several cleavage sites. Immunoblots with anti-OPA1 exhibited five protein bands (a-e). Proteases identified to be involved in OPA1 processing are m-AAA and PARL [34].

While the most prominent change was an increase of the expression level of band e with a moderate knockdown of prohibitins (shPHB and shPHB2-0), there was also an evident reduction of fragment a and b expression together with an increase of band e in shPHBz expressing cells, which showed a strong knockdown of prohibitins.

4.10 Mitochondrial networks

OPA1 is a mitochondrial fusion protein, and its knockdown leads to mitochondria fragmentation and loss of membrane potential[27]. The mitochondrial network was therefore examined in prohibitin knockdown cells. To this end, the mitochondria in prohibitin knockdown cells were immunofluorescently labeled with anti-Tom20 antibody. Pictures were obtained by confocal microscopy and mitochondrial structure was quantified with the ImageJ program[33]. Only the expression of shPHBz led to an increase in fragmented mitochondria (Figure 4-15).

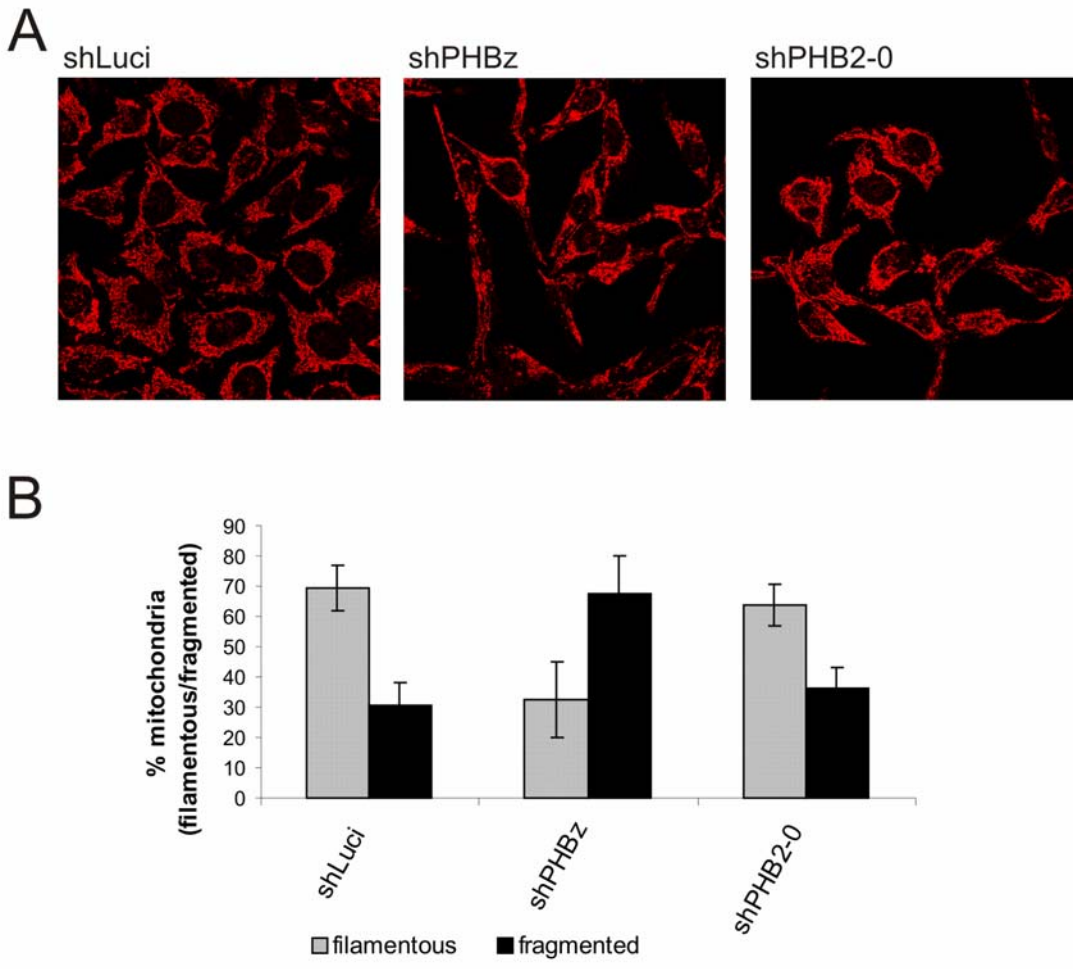

Figure 4-15: Expression of shPHBz resulted in an increase in mitochondria fragmentation
A, HeLa cells were seeded on slides after induction of the prohibitin knockdown for 12 days and mitochondrial structure was detected by immunofluorescence labeling with an antibody against Tom20. **B**. The mitochondrial structure was quantified by analyzing the pictures obtained by confocal microscopy with the ImageJ program. Only a prohibitin knockdown induced by shPHBz expression resulted in an increase in mitochondrial fragmentation.

This occurred in conjunction with a strong knockdown of prohibitin protein levels (Figure 4-16A), shown by western blot analysis of the cell lysates from the same

time point of induction. Additionally a change of the OPA1 expression pattern was verified and expression levels of the different isoforms was quantified (Figure 4-16B).

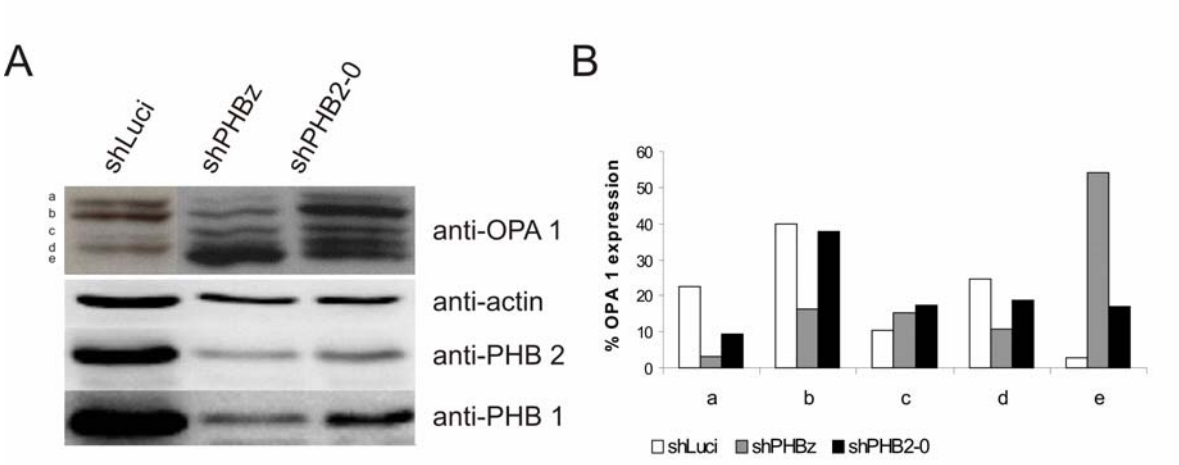

Figure 4-16: Mitochondrial fragmentation in shPHBz cells correlates with changed OPA1 pattern.
A, Cell lysates were taken from the same time point when mitochondria fragmentation (Figure 4-15) was assessed and probed for prohibitin and OPA1 expression by western blot. **B**, OPA1 expression was quantified with the AIDA program. The expression of band a and b was severely reduced in shPHBz cells, whereas it was only slightly reduced in shPHB2-0 cells. While the expression of band c and d was not significantly changed, expression levels of band e were increased 12 fold in shPHBz cells and 5 fold in shPHB2-0 cells compared to shLuci cells

Experiments depicted in Figure 4-15 and Figure 4-16 showed that prohibitin had an influence on mitochondrial structure and that certain phenotypes were dependent on the particular protein expression levels. Furthermore, the transition between mitochondrial fragmentation and OPA1 cleavage was smooth. The prohibitin knockdown in shPHB2-0 cells was comparable small and consequently only led to slight fragmentation of OPA1, while the time dependent increase in prohibitins reduction in shPHBz expressing cells resulted in increasing fragmentation of OPA1, followed by mitochondrial fragmentation.

However, the results presented also showed that the disrupted mitochondrial network was not the cause for the overall observed proliferation defect, as it only occurred in shPHBz cells, and was correlated with a severe reduction in prohibitin expression. The slackened proliferation rate of the cells already occurred when prohibitin levels were only moderately reduced.

4.11 Membrane potential

Reduced OPA1 expression by siRNA knockdown leads to the loss of mitochondrial membrane potential (MMP)[65]. To measure the MMP in prohibitin knockdown cells, the membrane potential sensitive dye TMRE was used and its fluorescence intensity was measured by FACS analysis. There was no loss or reduction of MMP in any prohibitin knockdown cells, irrespective of the knockdown intensity (Figure 4-17). As a control, the protonophore CCCP was added to each sample, as it effectively destroys the proton gradient through pore formation in the inner mitochondrial membrane. As shown in Figure 4-17, CCCP efficiently destroyed the membrane potential within 15 min of treatment.

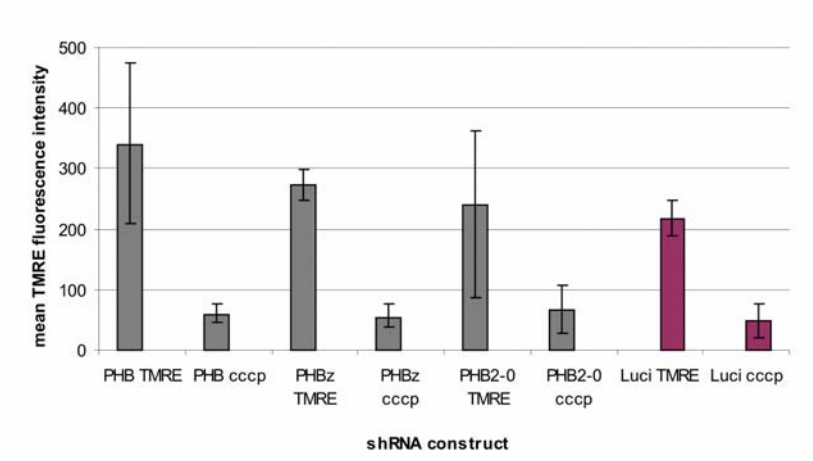

Figure 4-17: TMRE staining is not reduced in prohibitin knockdown cells
Mitochondrial membrane potential (MMP) was analyzed by staining prohibitin knockdown cells (10d p.i.) with TMRE. Fluorescence intensity was quantified by FACS and as a control 1µM CCCP was added to the already measured samples to destroy the existing MMP. Reduced prohibitin levels did not result in a loss of MMP.

Analysis of OPA 1 fragmentation of CCCP treated cells showed that, along with the loss of membrane potential, bands a and b were completely lost, whereas the expression levels of bands c-e are equally strong, indicating an increased expression of bands c and e (Figure 4-18).

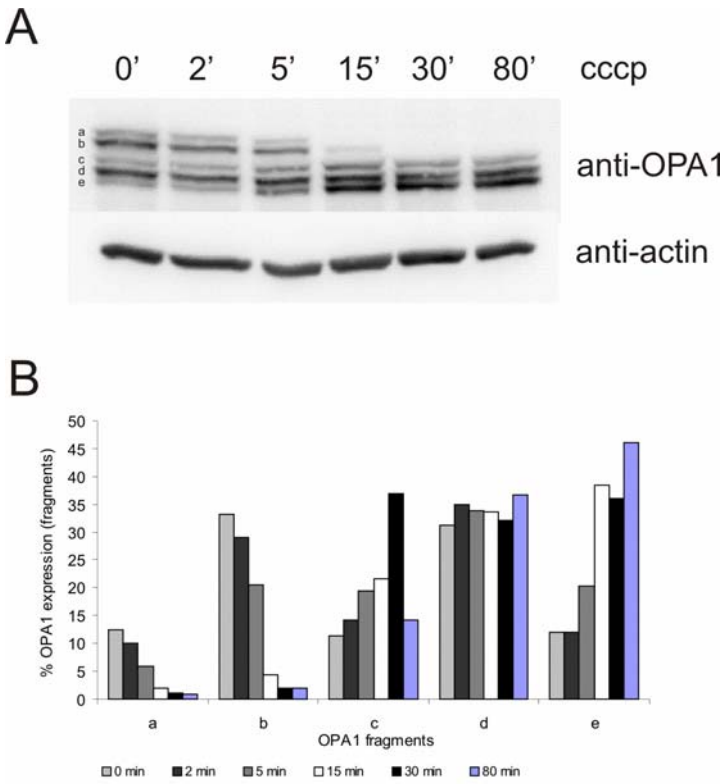

Figure 4-18: OPA 1 expression pattern was completely changed in CCCP treated HeLa cells
Ultimate fragmentation by dissipating MMP with 20µM CCCP for 15 min resulted in a complete loss of OPA1 protein fragments a and b as assessed by western blot while the expression level of bands c and e were increased to be at the same expression intensity as band d.

This phenotype was different to that seen in shPHBz expressing cells. An increasing loss of prohibitin protein levels never resulted neither in a complete loss of isoform b nor in an increase in expression level of isoform d.

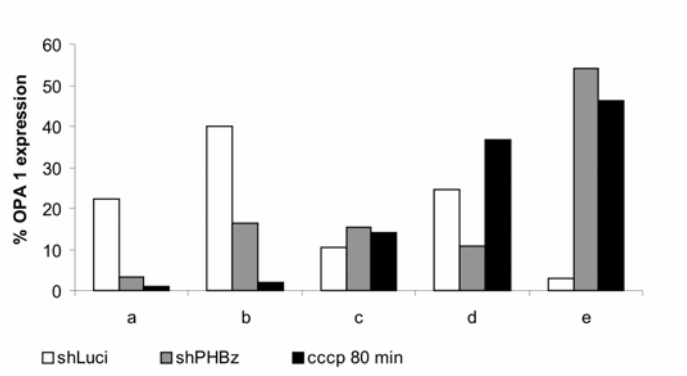

Figure 4-19: Comparison of OPA1 fragmentation in shPHBz cells and CCCP treated cells
CCCP treated cell and shPHBz expressing cells showed a different OPA1 cleavage pattern, with the fragmentation of OPA1 being more severe upon loss of MMP then upon reduced prohibitin protein expression.

4.12 EGFR overexpression

Within six to seven days of induction of a prohibitin knockdown, HeLa cells exhibited aberrant cell morphology, evidenced by reduced lamellipodia formation and the extension of long filaments (Figure 4-20).

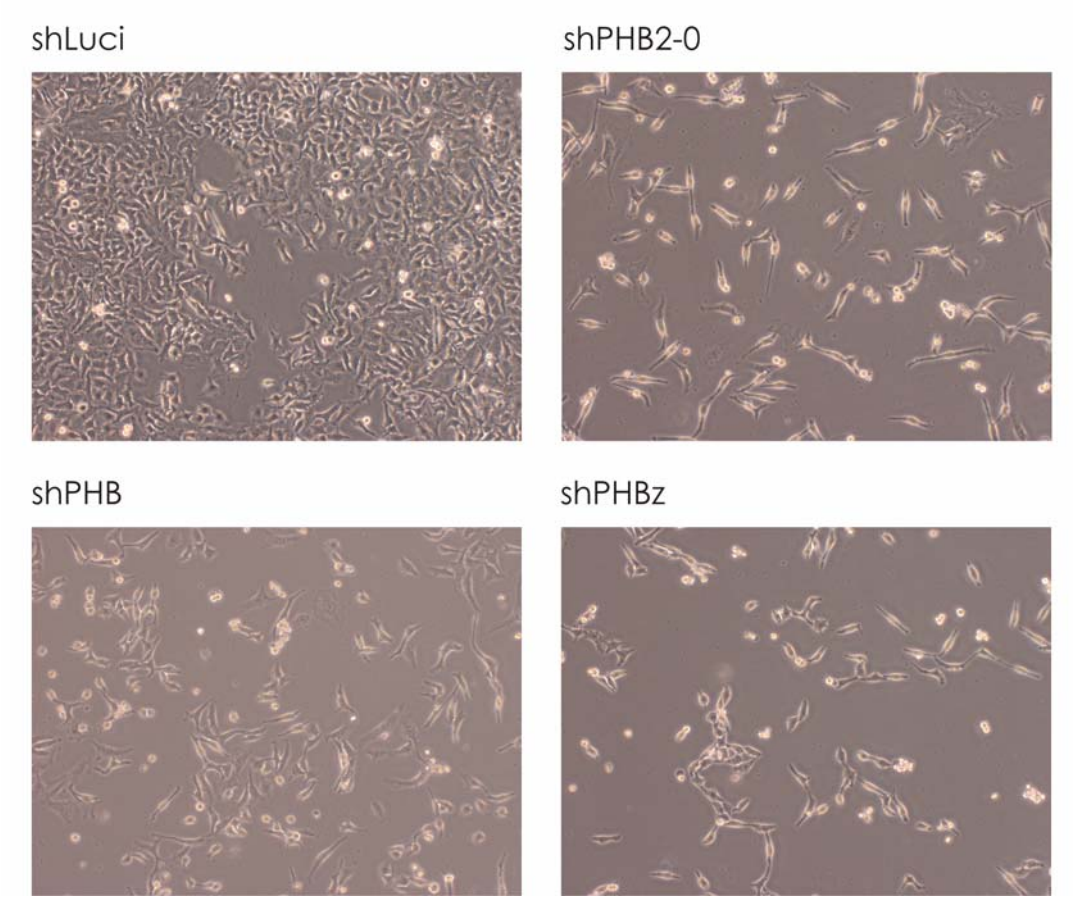

Figure 4-20: A prohibitin knockdown in HeLa cells leads to aberrant cell spreading
HeLa cells, induced for 10 days with doxycycline to suppress prohibitin gene expression (shPHB, shPHBz and shPHB2-0) show an aberrant spreading phenotype compared to induced shLuci cells. Prohibitin knockdown cells were attached to the plate only by their rear and front end, with the nucleus slightly detached.

This phenotype correlated with an increased expression of the epidermal growth factor receptor (EGFR) on RNA and protein levels (Figure 4-21A, B) as evaluated by qRT-PCR and western blot. To determine if this overexpression resulted in an increased cell surface expression of the protein, cells were subjected to extracellular immunofluorescence labeling with anti-EGFR, and fluorescence intensity was assessed by FACS analysis. The observed total EGFR overexpression was mirrored in a >2 fold increase of EGFR surface expression (Figure 4-21C).

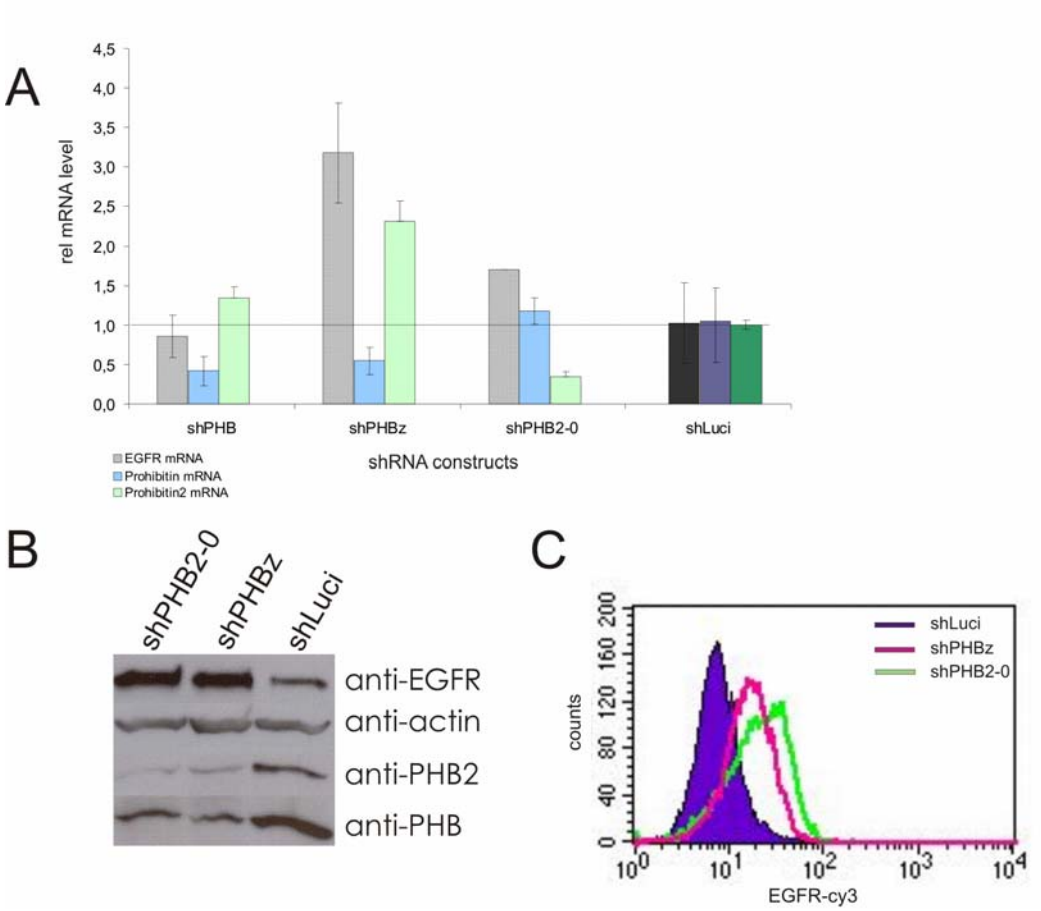

Figure 4-21: Aberrant spreading correlates with an overexpression of EGFR on the cell surface.
A, Prohibitin knockdown cells which displayed an aberrant spreading phenotype showed an increase of EGFR mRNA. This increase in mRNA level is reflected in EGFR protein level. **B**, Total EGFR protein level was assessed by western blot, using actin signal to normalize protein level. **C**, surface expression of EGFR was quantified by FACS analysis staining the protein with anti-EGFR and anti rabbit cy-3.

4.13 Cell-matrix /Cell-cell-contacts

Due to the aberrant cell spreading, the surface area of the cells was reduced. The adhesion of prohibitin knockdown cells to extracellular matrix was examined in the following experiments, to determine if a knockdown led to a decrease in cell-matrix contact formation. HeLa cells with a knockdown induced for 10 days were treated with CFSE to increase the fluorescence intensity to the measurable range of the microplate fluorometer. The cells were seeded for 30 min under growing conditions on fibronectin or BSA coated plates in order to wash off non-adherent cells. The number of adherent cells corresponded to the fluorescence intensity in each well. Cells featuring a knockdown in prohibitin proteins (shPHBz, shPHB2-0) showed a significant decrease in adhesion to fibronectin (Figure 4-22A).

Additionally, adhesion to collagen was reduced, as further demonstrated by the immunofluorescent labeling of cytoskeleton proteins in transduced HeLa cells.

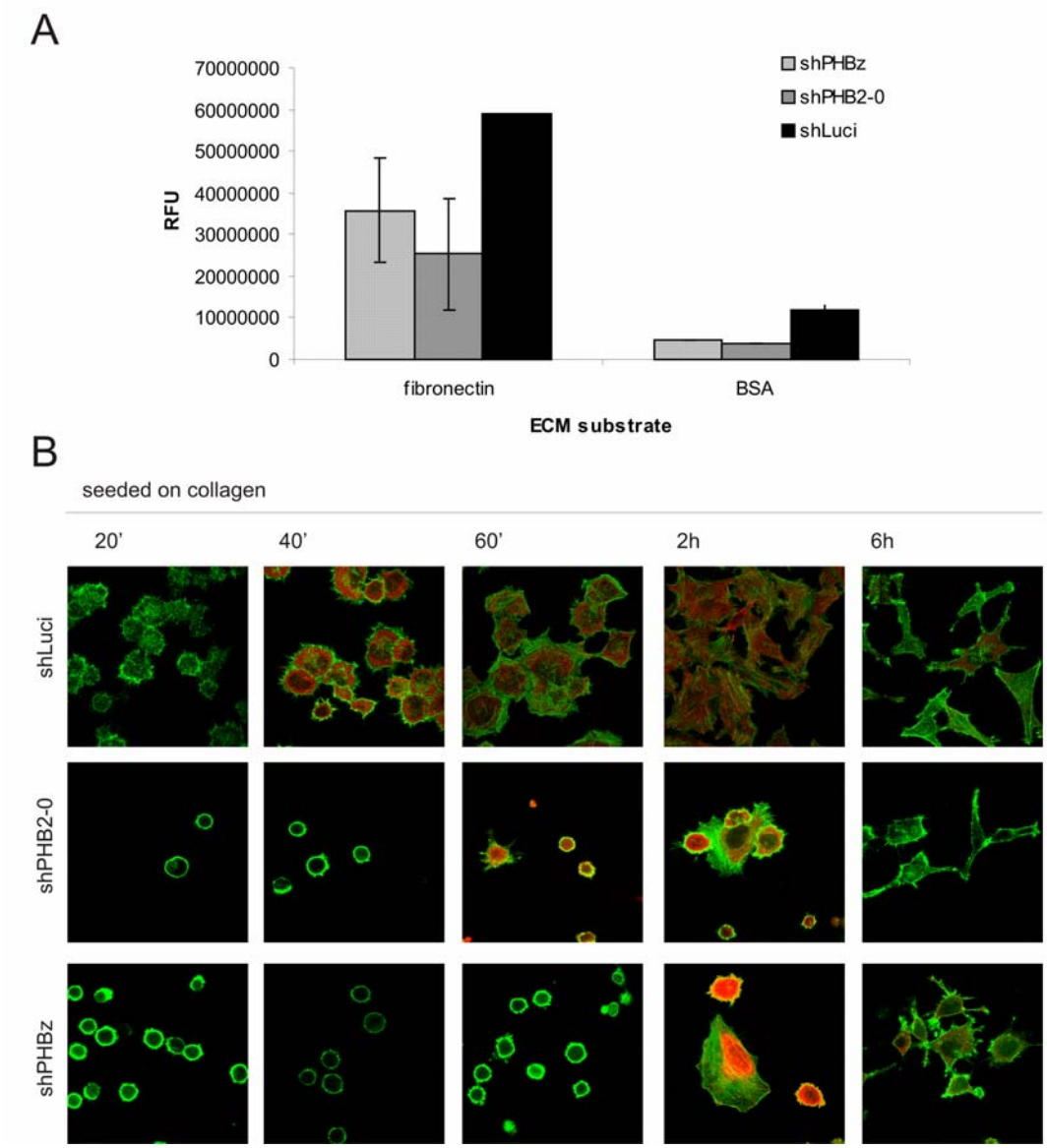

Figure 4-22: Prohibitin knockdown cells showed reduced adhesion to fibronectin and collagen
A, Prohibitin knockdown cells, induced for 10days, were treated with 5µM CFSE for 5min to increase the weak GFP fluorescence signal. The cells were then seeded on plates coated with 1mg/ml fibronectin or BSA, respectively and kept under growing conditions for 30 min. Non adherent cells were washed off with PBS and the amount of adherent cells was assessed by measuring fluorescence intensity. **B**, Prohibitin knockdown cells were seeded on collagen (2mg/ml) coated glass slides, kept under growing conditions for the indicated time. The slides were washed with PBS, fixed in 4% PFA and cytoskeleton proteins were stained with phalloidin-Alexa 488 (*green*) and anti-paxilin – anti mouse cy3 (*red*).

Therefore, HeLa cells induced for 10 days to express shRNAs, were seeded on glass slides coated with 2 mg/ml collagen. In a time course comprising five time-points cell adhesion capacity was analyzed by immunofluorescent labeling of the actin cytoskeleton as well as paxilin, a focal adhesion protein (Figure 4-22B).

Prohibitin knockdown cells showed reduced adhesion to collagen due to the longer time taken to form actin filaments and focal adhesions. While shLuci cells started to spread within 40 min of cell-matrix contact, cells with a prohibitin knockdown did not develop actin filaments and focal adhesion formation before 2h of attachment.

Both the formation of cell-matrix contacts and cell-cell contact formation were disturbed in cells with reduced prohibitin expression. This was observed by growing the cells under suspension conditions. While shLuci-HeLa cells formed colonies and grew slowly and steadily, shPHBz and shPHB2-0 HeLa cells did not form colonies and stopped proliferating (Figure 4-23).

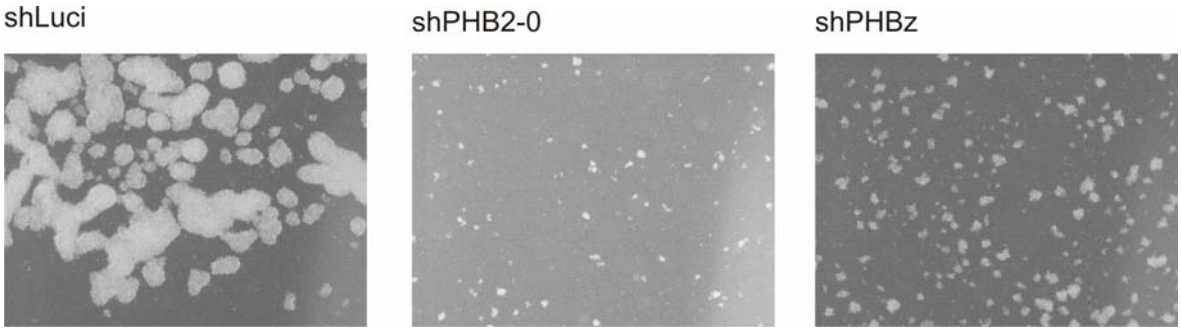

Figure 4-23: Under suspension conditions, prohibitin k.d. cells did not grow and form colonies.
Cell culture plates were coated with 1% agar to prevent attachment of cells, shPHBz, shPHB2-0 and shLuci induced HeLa cells, which were seeded in growth medium. Prohibitin knockdown cells showed a reduced proliferation and did not attach to each other to form colonies as did shLuci control cells.

These results clearly indicated that the proliferation defect observed in prohibitin knockdown cells is based on a defect in adhesion. Both cell-cell contact and cell-matrix formation are disrupted in these cells as the cells may have a defect in actin rearrangement and establishing cell polarity and this is necessary for cell division.