5 Discussion

Prohibitins have been identified as highly conserved proteins with multiple functions in cellular organisms. This diversified distribution complicates the allocation of these two proteins to specific cellular events, as the causes for occurring effects in overexpression or knockdown assays are manifold.

So far, most studies were restricted to overexpression studies with a knockout being proven lethal. With establishing RNA interference (RNAi) as a method to knockdown gene expression and therefore allowing the analysis of protein functions, siRNA approaches suggested roles for prohibitin in Ras/Raf/MEK as well as TGFβ signaling and identified it as a target gene of vitamin D in its function as a transcription factor. Nevertheless, an siRNA approach is restricted to a small range of transfectable cell lines and limits experimental procedures to four to six days of analysis. Thus, the generation of stable shRNAs expressing cell lines promised to expand the range of techniques. It would allow, for instance, the analysis of long term signaling events in cancer cells as well as extending the analysis of mitochondrial proteins to vertebrates, which has so far been limited to yeast.

5.1 Prohibitin stability

In this study, a set of specific shRNAs was used to generate cancer cell lines, soundly inhibiting prohibitin expression. While the mRNA knockdown was specific for the respective prohibitin, either 1 or 2, the loss of one protein by RNAi led to reduced protein levels of the other due to protein instability. Surprisingly, the mRNA of the prohibitin that was not down-regulated showed an increased expression level. Thus, mRNA levels of prohibitin 2 showed up to a 2.5 fold rise when prohibitin 1 mRNA was reduced by specific shRNA expression. This effect could be explained by an up-regulation of prohibitins' RNA expression under stress conditions, such as an infection with lentiviral particles. Presumably, the RNA expression levels of both genes get up-regulated, but as the mRNA of one is down-regulated by RNAi, only the increase in expression level of the other is visible. This would explain the difficulty in finding more than one functional shRNA to target prohibitin 1. Only 3 out of 14 tested shRNAs, designed following the Tuschl rules[85] resulted in a knockdown with more then 60% reduction in prohibitin mRNA levels. These were moreover targeting the coding region, shRNAs

designed to target the prohibitin 3'UTR were not functional. Hence, only highly effective shRNAs could compensate an increase in mRNA expression.

5.2 Proliferation defect

Prohibitin 1 was originally identified by its ability to inhibit G_1/S progression in human fibroblasts, a function which was later ascribed to its 3'UTR[42]. While some publications still consider the protein as a tumor suppressor, for instance as a regulator of E2F mediated transcription[89], most research groups concentrate on the mitochondrial function of prohibitin 1. Here, it has been suggested that prohibitin supports the import of respiratory chain complex proteins into the inner membrane[79].

In the present study it was shown for the first time that reduced prohibitin 1 expression led to slackened proliferation in various cancer cells. With the stable expression of shRNAs targeting prohibitin 1 mRNA, prohibitin expression was reduced up to 20 fold compared to control cells, but already weaker knockdown levels resulted in reduced proliferation. The field of prohibitin research is divided into those supporting the role of prohibitin as tumor suppressor protein and those supporting the role of prohibitin as a mediator of senescence and inhibitor of apoptosis. Its role as a tumor suppressor was ascertained with research methods confined to overexpression studies using cDNA or mRNA[38; 52; 88], while prohibitins function in senescence and apoptosis was identified in yeast or using siRNA transfection approaches, respectively[68; 69]. In this work, using an shRNA expression approach, prohibitin was identified as a mediator of proliferation. This clearly showed that the choice of method is critical for the identification of protein function. The data presented here are in consistence with knockout studies in yeast, where the deletion of prohibitin leads to a decrease in the replicative life span[18]. Moreover, previous siRNA transfection studies showed a sensitization towards apoptosis, a phenotype not expected from a tumor suppressor protein[43; 69]. Overexpression studies not only identified an unlikely localization for prohibitin 1 in the nucleus but assigned it a function as regulator of transcription *via* interaction with pRb and E2F[29; 88]. None of the performed experiments in the present study allowed identification of a nuclear function for prohibitin 1. Neither was overexpressed GFP-tagged prohibitin found anywhere else but in the mitochondria, nor did the immunofluorescenct labeling of endogenous prohibitin

reveal a nuclear localization, considering that common available antibodies are not very efficient in labeling the endogenous protein (data not shown). Furthermore, the published results on E2F and prohibitin interaction were based on simple purification of prohibitin proteins from *E.coli.* It is common knowledge in this field that prohibitin proteins are accumulated in inclusion bodies and can not be purified without denaturation procedures[60]. Therefore it is highly questionable that Chellappan *et al*. identified a function for prohibitin 1 protein as a tumor suppressing.

Although prohibitin knockdown cells have a clear defect in cell division, a block at any of the cell cycle check points could not be detected. Neither could a senescent status be made responsible for this phenotype, as staining for SA-β-galactosidase did not give positive results. A potential block in mitogenesis, which would not be detected with a BrdU assay, as rounded cells would be washed off during the labeling procedure, could be excluded; analysis of the CFSE distribution clearly showed that the cell division process was simply slower. Different signaling pathways and complexes known to involve prohibitin 1 were analyzed. As none of them, neither Ras/Raf/MEK signaling nor the function as mitochondrial chaperone with respect to ATP synthesis and membrane potential, was unchanged, the cause of the slackened proliferation was ascribed to a defect in the adhesion ability of the cells

Due to the observed proliferation defect, constitutive shRNA expression did not resolve the limitations of the siRNA transfection approach. Hence, an inducible shRNA expression system was established, which allowed a prohibitin knockdown to be brought on at any given moment by administering doxycycline. This approach allowed, for example, growth of the experimentally required amount of cells. To obtain comparable results, experiments were done after ten days of induction with doxycycline.

5.3 Adhesion/migration

HeLa cells that constitutively expressed shPHBz and shPHB2-0 as well as HeLa cells that exhibited reduced prohibitin protein levels upon induction with doxycycline, showed a severe adhesion defect. Furthermore the propagation of prohibitin knockdown cells for more than six days resulted in changed morphology. The formerly nearly fibroblast-like phenotype was changed into a stretched form,

with the cell only attached to the plate by the rear and front end, and with the nucleus slightly detached. This phenotype is reminiscent of the hummingbird phenotype seen with *H.pylori* infection, where it is ascribed to activation of c-met receptor, or in cells expressing a mutant, permanently active EGF-receptor (EGFR)[6; 16; 62]. Excitingly, this phenotype, observed in prohibitin knockdown cells, correlated with an overexpression of EGFR on the surface. However, an increased activation of EGFR itself, measured by p-EGFR antibody staining on western blot, was not observed. Neither was an increase in EGFR signaling detected, as assessed by Erk activation. Besides activation of the mitogen-activated protein kinase (MAPK) pathway, EGFR signaling is tightly connected to integrin receptor signaling and an overexpression often goes hand in hand with a changed surface expression of integrins[62; 93]. Therefore, the observation that integrin mRNA levels were regulated in prohibitin knockdown cells, as analyzed by qRT-PCR and a microarray, endorsed the impression that the loss of adhesion was cause for the observed proliferation defect.

Integrins promote adhesion *via* cell-matrix contacts[32]. The importance of anchorage for proliferation becomes apparent, when anchorage independent growth leads to malignancy, a hallmark of cancer cells as described in chapter 2.2. Loss of adhesion, acquired through the absence of substratum leads to reduced transcription and translation[7]. Moreover, following a block in cyclin D1 activation and consequently block in Rb phosphorylation, non-transformed cells arrest in the G_1-cell cycle phase upon loss of adhesion[9]. Cyclin D1 and A up-regulation can eventually be a result of integrin receptor signaling[9; 94]. However, the finding that a reduced block in adhesion leads to an arrest in G_1/S-progession is not consistent with the results obtained in the present study. Although BrdU uptake was reduced in prohibitin knockdown cells, a sign for a block in S-phase progression[22], a block at a G_1 checkpoint by analysis of the DNA content using PI staining could not reproducibly be identified. Nevertheless, the CFSE staining, visualizing the cell division by measuring cytoplasm distribution to the daughter cells, clearly showed a reduced proliferation rate. As the cells were not devoid of prohibitin proteins, with shRNA expression only leading to a reduced expression, there was probably only a slight block in cell cycle progression, which was not detectable with a crude PI staining. As it is the spreading of the cell that promotes proliferation and not adhesion per se[11; 25], additional effects might have promoted the proliferation

defect. This was supported by the finding that not only integrins were regulated on mRNA level but extracellular matrix (ECM) proteins like collagens and fibronectins as well. Furthermore not only cell-matrix contacts were aberrant from prohibitin knockdown cells, but also the formation of cell-cell contacts, mediated by members of the cadherin and catenin family. However, it is not much known about proliferation control through cell-cell contacts [50; 78].

Common between cell-matrix contacts and cell-cell contacts is the polymerization of actin near the side of receptor activation. This local activation of actin polymerization, enhanced by microtubule orientation, leads under endogenous circumstances to a polarization of the cell. This process is necessary for migration and proliferation, which are both inhibited in prohibitin knockdown cells[23].

The conclusion to be drawn from this was that a prohibitin knockdown disrupts the polarization of the cell, maybe due to incomplete actin polymerization and microtubule reorientation, which in consequence led to the observed defects in migration and proliferation. Further experiments should include the analysis of integrin expression levels on the surface and the verification of a potential block in G_1/S-phase progression.

An important information to consider in further experiments is the fact that doxycycline is a broad spectrum inhibitor of matrix-metalloproteases[30]. These proteases cleave extra-cellular matrix proteins and therefore allow cell migration. The phenotype observed in prohibitin knockdown cells might therefore be an artifact, ascribed to the effectiveness of doxycycline. This is contrary, though, to the facts that, firstly, shLuci cells were induced for the same length of time and did not display a changed morphology and, secondly, that the used concentration of doxycycline (1 µg/ml, approximately 2 µM) is more than 50 times below the tested concentration of 100 µM, which was furthermore used in smooth muscle cells[26]. Although HeLa cells infected with the non-inducible shRNA expression system did not display such a severe phenotype, this absence of an effect could be imputed to the length of a prohibitin knockdown, which could not be reached with the non-inducible system due to the proliferation defect.

5.4 Prohibitin 3'UTR

As already discussed in chapter 5.2, the cells with reduced prohibitin protein expression clearly exhibited a slackened proliferation rate. Therefore, the notion that prohibitin 1 protein is a tumor suppressor should be reconsidered. In addition, the 3'UTR of prohibitin 1 was predicted to have anti-proliferative function in cancer cells, particularly breast cancer cells that are homozygous for the T-allele. Different from the C-allele, the T-allele exhibits a specific single nucleotide polymorphism (SNP), a point mutation from C->T, at position 729bp of the 3'UTR[39]. HeLa cells, although not a breast cancer cell line, were allocated to carry the T-allele[40]. However, several findings presented in this work questioned the assumption that prohibitins 3'UTR carries tumor suppressing function. Transfection of the non-mutated 3'UTR into cells only expressing the mutated T-allele was expected to block proliferation. This was not observed in HeLa cells. Furthermore, sequencing of the 3'UTR of a set of cell lines revealed a range of point mutations but only limited to cancer cell lines. A transformed primary cell line, IMR-E1A, had no point mutation. Why the tumor suppressing function should then be limited to the expression of the C-allele is questionable. Furthermore, using the technique of RNAi to reduce protein expression automatically reduced mRNA expression of the protein of interest, including the 3'UTR. Loss of a tumor suppressor, be it RNA, should increase proliferation. This was, as well, not observed in shRNA mediated prohibitin knockdown cells.

For the 3'UTR of prohibitin 2 no function is described. Based on experiments done in our group, the prediction could be made that the 3'UTR of prohibitin 2 codes for a micro-RNA. Loss of this micro-RNA would explain the differences observed in the knockdowns *via* shPHBz and shPHB2-0 expression. Although the prohibitin knockdown was much more severe in shPHBz cells, HeLa cells expressing shPHB2-0 were increasingly sensitized towards apoptosis. These cells were lost due to cell death within ten days of knockdown induction, while shPHBz cells were not affected. To test if the prohibitin 2 3'UTR encodes for a micro RNA, further experiments should include the transfection of the 3'UTR into shPHB2-0 knockdown cells. If the reduced expression of a specific micro RNA was cause for the observed cell death, reexpression of the 3'UTR is expected to rescue the shPHB2-0 cells from apoptosis.

5.5 Mitochondrial chaperone

Research done in yeast showed that prohibitins are necessary for effective import and integration of the members of the respiratory chain complexes into the inner membrane. Prohibitins form a complex with the m-AAA protease, which degrades incorrectly folded proteins from the respiratory chain complex[49; 79]. While a deletion of the m-AAA protease in yeast leads to an impaired degradation of non-assembled inner-membrane proteins, the deletion of prohibitin proteins leads to an increased turnover in respiratory chain complex proteins[79]. This was not observed in the shPHBz or shPHB2-0 background of shRNA expressing cells. Different proteins of the respiratory chain were analyzed, as antibodies were available, but no change in expression levels was detected. Moreover, loss of ATP synthesis, expected in case of an impaired assembly of the respiratory chain complex, was not detectable. In fact, for the expression of shPHBz, resulting in the strongest prohibitin knockdown of the used shRNAs, a slight increase in ATP synthesis was detected. At the same time, the mitochondrial membrane potential (MMP), established through proton transfer via the respiratory chain was not decreased. This further indicates that the import of respiratory chain complex proteins into the mitochondrial inner membrane was not impaired when prohibitin expression was only reduced and not lost. As prohibitin mRNAs are presumably up-regulated under stress conditions, it might be possible that an effect of a prohibitin knockdown on proteins of the respiratory chain is only visible with an increase of stress factors. Moreover, it is not clear yet, if it is only an inhibitory influence that prohibitins exert on the m-AAA protease or if they have an active role in promoting integration into the inner membrane[79]. As the m-AAA protease degrades non-assembled proteins, probably only their accumulation, for instance through an increase in reactive oxygen species (ROS), together with a knockdown in prohibitins, would lead to a visible defect in mitochondrial protein composition or the loss of ATP synthesis. Therefore, it remains to be seen, whether stress induction leads to a severe mitochondrial defect in prohibitin knockdown cells.

Although a loss of MMP was not detectable, with the strong reduction of prohibitin expression, mitochondria were fragmented. Analysis of the mitochondrial fusion protein OPA1 revealed that its expression was not lost from prohibitin knockdown cells as published by Kasashima and colleagues[43] but that the pattern of the five visible isoforms was changed, displayed through the reduction in band a

and b levels and an increase in band e expression. With the induction of the ultimate fragmentation by dissipating MMP, bands a and b were aberrant and bands c-e equally distributed. This observation confirmed the findings from Ishihara *et al.*[34] that it is the loss of the large OPA1 fragments, band a and b, that leads to mitochondria fragmentation and not necessarily the complete loss of OPA1 expression, e.g. induced by RNAi. These results further indicated that mitochondrial fragmentation is a process with smooth transition, as a moderate prohibitin knockdown in shPHB2-0 cells exhibited the beginning of OPA1 fragmentation visible by an increase in band e levels but did not feature fragmented mitochondria yet.

This effect of prohibitins on mitochondria morphology could be explained by a possibility that a protease, which is responsible for OPA1 cleavage, was affected. With PARL and m-AAA protease, two mitochondrial proteases have been predicted so far to have functions in the processing of OPA1. While Pcp1p, the yeast orthologue to PARL has been shown to be the only protease to process OPA1 in yeast, in vertebrates it is still highly debated which and how many proteases are involved in its maturation and processing. By means of overexpression studies it was shown that OPA1 is solely processed by m-AAA protease[34]. The role of PARL is limited to the processing of a fusion-incompetent, antiapoptotic fragment, which is not part of the five band OPA1 expression pattern[27]. The results obtained with an shRNA mediated prohibitin knockdown now allowed assuming that at least two proteases are involved in the generation of the endogenous OPA1 pattern (Figure 5-1). The prohibitin knockdown led to a highly reduced expression of the band a with a concomitant increase in the band e. Contrary to the OPA1 expression pattern seen with CCCP treatment, the band b was still consistently expressed in prohibitin knockdown cells even though with a slightly reduced level. This appearance was accompanied by reduced band d expression, again different from the one seen in CCCP treated cells, where band d levels were increased. This indicates that the band b is subject to the processing of a second protease. As the studies limiting OPA1 processing to m-AAA protease activity were done by overexpressing two out of eight splice variants, it is not unlikely that further splice variants are processed by a different protease. Furthermore, studies using a PARL knockout to analyze OPA1 cleavage did not

distinguish between the different expressed isoformes, therefore a possible loss or increase of one isoform through PARL cleavage should not be excluded.

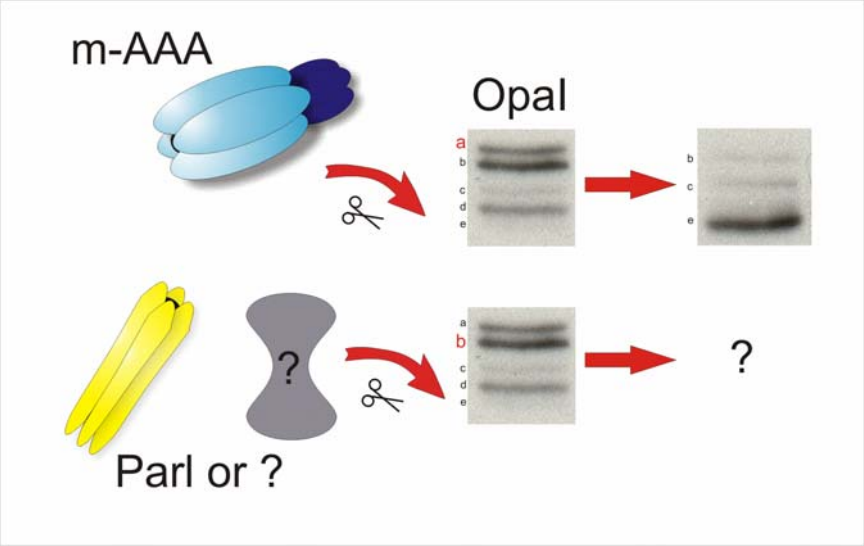

Figure 5-1: Processing of OPA1 by m-AAA and Parl or a 3rd unknown protease

Prohibitins clearly play a role in maintaining mitochondrial morphology, most likely by regulating proteases in the inner mitochondrial membrane. As numerous studies in yeast have shown an interaction of prohibitins and m-AAA protease, their chaperoning function is, considering the presented results, likely to be extended to vertebrate cells. By using shRNA mediated RNA interference, it was shown that mammalian mitochondria are an equally good model system as the yeast mitochondria are. This technique can therefore enable the identification of the OPA1 processing proteases in vertebrates and thereby facilitate the identification of the different functions of the OPA1 isoforms. Further applications for an shRNA mediated knockdown of mitochondrial proteases like m-AAA and PARL could be the identification of other mammalian target proteins processed by these proteases, for instance using 2DE SDS-Page.

The application of RNAi to induce a stable knockdown of prohibitin proteins in mammalian cells allowed the identification of prohibitins' role in adhesion. It is the loss of adhesion and cell contact formation that reduced the rate of cell proliferation in prohibitin knockdown cell. However, it remains to be shown, how prohibitins are involved in regulating the rearrangement of the cytoskeleton.