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Faculty of Science

Department of Molecular Biology and Biochemistry

Master thesis

Functional analysis of prohibitin in *Trypanosoma brucei*

Jiří Týč

Supervisor: Prof. RNDr. Julius Lukeš, CSc.
Institute of Parasitology, Academy of Sciences of the Czech Republic
Laboratory of Molecular Biology of Protists

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Anotation:
In this study the importance of prohibitin1 and prohibitin2 genes for *Trypanosoma brucei* was examined. RNA interference showed that they are essential for parasites to survive. Knocking down of these genes resulted in altered morphology of the mitochondrion, changes in membrane potential and shut down of mitochondrial translation. No changes were observed in levels of Reactive Oxygen Species and respiration. Both prohibitines are part of big complex present in the mitochondrion.

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I hereby declare that I did all work, summarized in this thesis, on my own or in collaboration with co-authors of the presented paper, and only using the cited literature.

Prohlašuji, že v souladu s § 47b zákona č. 111/1998 Sb. v platném znění souhlasím se zveřejněním své Diplomové práce, v úpravě vzniklé vypuštěním vyznačených částí archivovaných Přírodovědeckou fakultou, elektronickou cestou ve veřejně přístupné části databáze STAG provozované Jihočeskou univerzitou v Českých Budějovicích na jejích internetových stránkách.

České Budějovice, April 20, 2010

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Jiří Týč

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Of course I want to thanks all the other members of our lab: Hassan, Zdeňka, Zdeněk V., Zdeněk P., Pavel, Petr, Lucka N., DHL, Tomáš, Shao, Lucka H, Pasha and Ola, Som, Piya, Gabča, Jirka, Alča, Brian, Míša and Karolína for their everyday regular help and understanding. I cannot forget all my good friends and last but not least - my family because without their support I would never reach this point in my life.

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a) Article

I) Paper's Preface

I am the first author of the article:
Jiří Týč, Drahomíra Faktorová, Eva Kriegová, Milan Jirků, Zuzana Vávrová, Dmitri A. Maslov & Julius Lukeš (2010) Probing for primary functions of prohibitin in Trypanosoma brucei. Int. J. Parasitol. 40, 73-83.

My contributions were as follows:
I performed the transmission electron and fluorescence microscopy experiments. I carried out the cell fractionation and measurements of membrane potential, reactive oxygen species, and respiration. To all of these events (except the cell fractionation) I assigned the time point at which they occur. All of these experiments were done simultaneously on all three different cell lines available: 1) PHB1 knockdown using p2T7-177 vector, 2) PHB1 knockdown using pLew100 vector and PHB1+2 doubleknockdown using p2T7-177 vector. I also repeated the growth curve experiment. I have also prepared all pictures for the publication.

Probing for primary functions of prohibitin in *Trypanosoma brucei*

Jiří Týč[a], Drahomíra Faktorová[a], Eva Kriegová[a], Milan Jirků[a], Zuzana Vávrová[a], Dmitri A. Maslov[b,*] & Julius Lukeš[a,*]

[a]Biology Centre, Institute of Parasitology, Czech Academy of Sciences, and Faculty of Natural Sciences, University of South Bohemia, České Budějovice (Budweis), Czech Republic
[b]Department of Biology, University of California, Riverside, CA 92521, USA

ABSTRACT

Prohibitins (PHBs) 1 and 2 are small conserved proteins implicated in a number of functions in the mitochondrion, as well as in the nucleus of eukaryotic cells. The current understanding of PHB functions comes from studies of model organisms such as yeast, worm and mouse, but considerable debate remains with regard to the primary functions of these ubiquitous proteins. We exploit the tractable reverse genetics of Trypanosoma brucei, the causative agent of African sleeping sickness, in order to specifically analyse the function of PHB in this highly divergent eukaryote. Using inducible RNA interference (RNAi) we show that PHB1 is essential in T. brucei, where it is confined to the cell's single mitochondrion forming a high molecular weight complex. PHB1 and PHB2 appear to be indispensible for mitochondrial translation. Their ablation leads to a decrease in mitochondrial membrane potential, however no effect on the level of reactive oxygen species was observed. Flagellates lacking either PHB1 or both PHB1 and PHB2 exhibit significant morphological changes of their organelle, most notably its inflation. Even long after the loss of the PHB proteins, mtDNA was unaltered and mitochondrial cristae remained present, albeit displaced to the periphery of the mitochondrion, which is in contrast to other eukaryotes.

Keywords: Prohibitin, Trypanosoma, Mitochondrion, Morphology, Mitochondrial translation

b) Recent data

7) DISCUSSION

Prohibitins (PHBs) are conserved and throughout eukaryotes represent a family of ubiquitously expressed membrane proteins for which various roles in different cellular compartments have been assigned. Here I want to briefly review and discuss some of the new data that appeared in the literature since we have published our results.
Our paper was accepted on 10 July 2009, was available on the internet since August 2009 and finally came out in the printed form in January 2010.
Since then, several papers have been published on the prohibitin subject. Connection between PHB2 and breast cancer through estrogen receptor signaling was examined by (Kim et al., 2009). Here the function of PHB2 as a nuclear transcription inhibitor was described in more details. Connection of PHBs with cancer and some other mainly neuro- and muscular degenerative (those are obviously mainly connected to PHBs mitochondrial function) diseases were known for some time already. Very recently PHBs were linked even to AIDS. With the PHB1 and 2 heterocomplex in the cytosolic membrane being capable of binding to HIV-1 glycoprotein (Emerson et al., 2010).
Sikora et al., 2009 in their proteomic analysis in yeast affected by cytosolic [PSI+] prion (self perpetuating conformation of the translation termination factor Sup35) identified PHBs as one of 44 proteins, the levels of which were affected by this prion. Surprisingly this cytoplasmatic prion has a strong influence on the mitochondrion. Authors link some of the detrimental effects of this prion right to the PHBs unnaturally accumulated in the cytosol, which impaired their mitochondrial function. One other protein identified in this study was Cox2 (mitochondrially-encoded protein). The authors propose that actually the absence of PHBs is potential reason of Cox2 destabilization (Sikora et al. 2009). That assertion would be consistent with our findings that mitochondrial translation is affected in PHBs deficient cells. Another phenotype in PSI affected yeast cells was mitochondrial fragmentation – this phenomenon was also connected to the PHBs via the OPA1 proteins (Merkwirth et al., 2008). Moreover, a connection between the prohibitin complex and mitochondrial m-AAA (matrix-ATPases associated with diverse cellular activities) proteases in yeast has been known since 1999 (Steglich et al. 1999) (FIG. A). Recently the same connection was confirmed in *Arabidopsis thaliana* (Plechota et al., 2010). This work revealed complexes of prohibitins and two different m-AAA proteases - AtFtsH3 and AtFtsH10, both of them being able to form homo- and heterocomplexes with prohibitins. Contrary to yeast and humans, the formation of the m-AAA complexes in *A. thaliana* is strictly dependent on the PHB complex. Thus in this model organism prohibitins may be considered a kind of scaffold for m-AAA proteases (Plechota et al., 2010).
From the model organism *Caenorhabditis elegans* new data about prohibitins capacity to promote longevity and affect energy metabolism and fat utilization have been published in Nature (Artal-Sanz and Tavernarakis, 2009a). Emerging evidence that PHBs may actually function as protein and lipid scaffolds that ensure the integrity and functionality of the mitochondrial inner membrane are also discussed in this study.

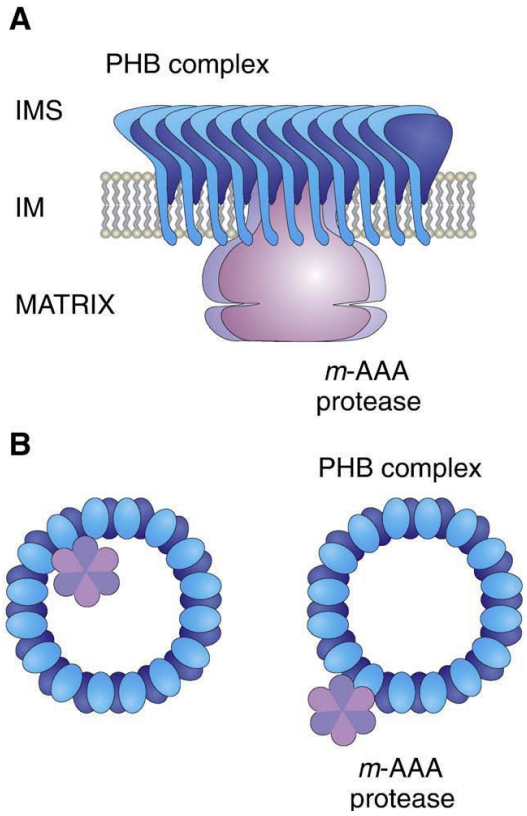

Fig. A. (Merkwirth and Langer 2009)
Supercomplex of prohibitins with the ATP-dependent m-AAA protease. In contrast to prohibitins, m-AAA protease subunits expose their catalytic domains to the matrix space. The binding of the m-AAA protease to the inner or outer surface of ringshaped prohibitin complexes remains to be established. (A) Side view of the assembledsupercomplex. (B) Potential arrangement of prohibitins and m-AAA protease within thesupercomplex. IMS = intermembrane space, IM = inner membrane.

Furthermore Artal-Sanz and Tavernarakis, 2009b published a review focused on prohibitin in relation with its mitochondrial functions. But nothing strikingly new came out. Prohibitins remain connected to many important processes in the cell (for details see introduction of our article), but still indirectly and the process of their function remains elusive (Fig B).

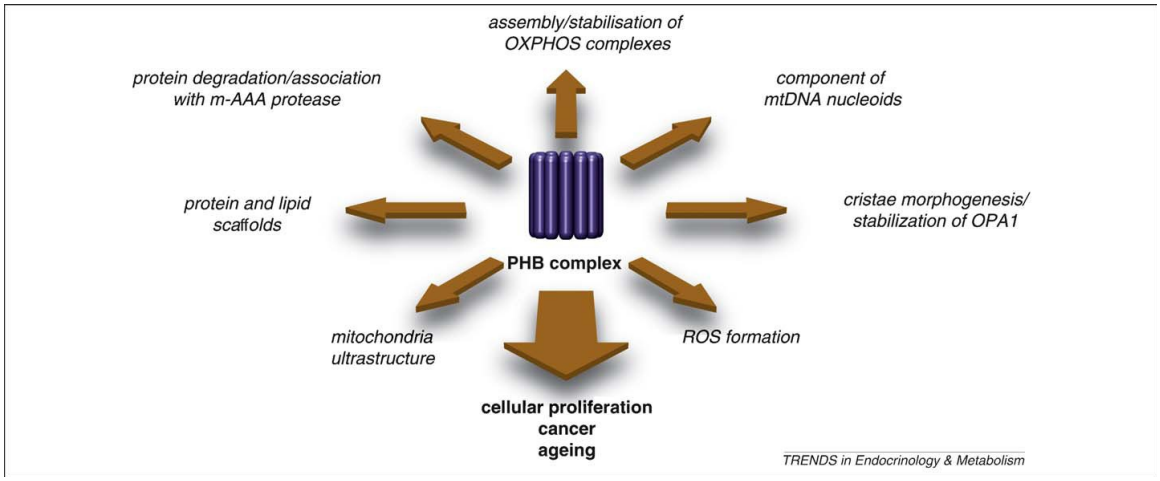

Figure B. (Artal-Sanz and Tavernarakis, 2009b) Involvement of the PHB complex in mitochondrial biology and cellular function. The PHB complex has been proposed to play diverse roles within mitochondria (indicated by arrows). Although the exact mechanism of action of prohibitins remains unknown, the pronounced effects of prohibitin depletion in various organisms highlight the importance of this evolutionarily conserved PHB protein complex.

The most interesting paper for us is that of Jain et al., 2009, not just for citing our work, but more importantly, for their model organism *Leishmania donovani*. This parasitic protist belongs into the same group - Kinetoplastida - as *Trypanosoma brucei* studied by us. Surprisingly in *L. donovani* prohibitin seems to be almost exclusively in the cell plasma membrane. This is quite surprising since in the case of *T. brucei* we did not find any sign of localization of PHBs outside of the mito-

chondrion. Moreover our data correspond well with the literature (Merkwirth and Langer 2008) that PHBs are originally mitochondrial proteins of all eukaryotes including protozoa, which only later gained in multicellular organisms a new function, perhaps in frame of cell-to-cell communication and other processes. In any case connection with plasma membrane is not that new for prohibitins as for example PHB2 was described as B-cell receptor associated protein (Terashima et al., 1994) and both PHB1 and PHB2 function in human intestinal epithelial cells as a binding site for the capsular polysaccharide of *Salmonella typhi* (Sharma and Aadri, 2004).

Jain et al. (2010) showed that in *L. donovani* prohibitin is involved in binding of the parasite to the host cell where it actually binds to the HSP70 protein, so once again PHB functions in cell to cell interaction. Our and Jain et al. (2010) data are not in conflict, as the latter authors were able to detect some signal, although not very strong, in the mitochondria.

Why did not we find PHBs in the plasma membrane of *T. brucei*? Here I propose some possible explanations. There is a major difference between the life cycles of *Leishmania* and *T. brucei*. Procyclic stages of both pathogens live in the gut of their insect vector, but while *T. brucei* lives in blood of its mammalian host (Matthews 2005), *Leishmania spp* are intracellular parasites (Cunningham 2002). Thus *Leishmania* needs some proteins for binding to the host cell - why not to use among the other proteins the preadapted PHB? Another thing is that Jain et al. (2010) were primarily focused on host parasitic interaction, so the metacyclic stage (the one that actually comes to interaction with the mammalian host) of *Leishmania* was used for most experiments. On the contrary we have worked with the procyclic stage of *T. brucei*, because we were focused on the mitochondrion. Another practical aspect of this line of research was that the bloodstream stage has a highly reduced mitochondrion which is not easy to work with. Since there are big changes not only in morphology but also in metabolism and membrane composition between the stages in both *Leishmania* and *T. brucei* (Yao et al. 2010, Vertommen et al. 2007), there can actually be major difference between stages of prohibitin function and abundance.

There is actually a report that both PHB1 and PHB2 are present in the *T. brucei* flagellar proteome (Broadhead et al. 2006). Since this results were obtained from mass spectrometry analysis of isolated flagella, contamination with other cell fractions is likely. What is more, this experiment was done with the bloodstream stage of *T. brucei*.

At the end of their discussion Jain et al., (2010) propose: "...that the evolutionarily conserved protein prohibitin shows a totally different function in *Leishmania* unlike the higher eukaryotes. It serves as an important entity in host–parasite interactions and can be viewed as a target for drugs or a diagnostic marker." I do believe their experiments and results. But personally I would question this statement. The participation of PHB in *L. donovani* in the attachment process to the host cell is unique but as I mentioned above, PHBs in general do occur in plasma membrane in cell-to-cell interaction and *L. donovani* simple used this preadaptation of this protein in its own way. Also the mitochondrial function of PHB was not totally excluded.

In *Leishmania major* prohibitin (LmjF16.1610) was found as a metacyclic-specific gene during analysis of cDNA microarrays search for stage specific genes (Almeida 2004). This gene was actually used by Jain et al., (2010) for designing primers against *L. donovani*. The same as all other eukaryotes *L. major* has two prohibitin genes - already mentioned LmF16.1610 and LmF35.0070. Prohibitins, with some exceptions (Rajalingham and Rudel, 2005) usually work together in a large complex both in the mitochondrion (Merkwirth and Langer 2009) (Fig. C) and also in plasma membrane (Sharma and Aadri, 2004). For their mitochondrial function cooperation between PHB1 and PHB2 is essential. So I wonder why Jaine et al., (2010) did not mention this second prohibitin at all? It would be interesting to know, what is the localization of this second prohibitin protein in *L. donovani*. It may give us the answer, whether or not are PHBs in the complex in its mitochondrion as everywhere else. The question, whether single PHB or the complex is involved in the process of *L. donovani* and the mammalian host cell might also be of some value.

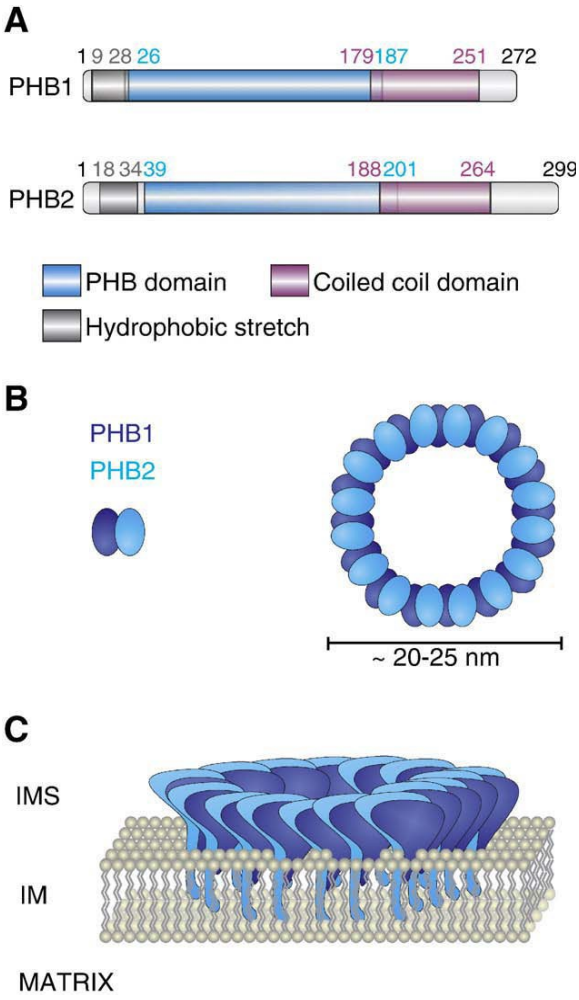

Fig. C. (Merkwirth and Langer 2009) Complex assembly of prohibitin subunits in mitochondria. Schematic representation of prohibitin subunits PHB1 and PHB2, the ring-shaped prohibitin complex and its topology in the mitochondrial inner membrane. (A) Domain structures of mammalian prohibitins. Gray boxes indicate hydrophobic stretches; blue, PHB domains (also termed SPFH domains); violet, coiled-coil domains. Numbers in corresponding colours refer to the respective amino acid residues in murine PHB1 and PHB2. (B) Dimers of PHB1 and PHB2 as building blocks of prohibitin complexes. Heterodimers assemble into ring-like prohibitin complexes with alternating subunit composition. The average stoichiometry of the complex is speculative. The average diameter of ring complexes is ~20–25 nm. (C) The prohibitin complex is anchored to the mitochondrial inner membrane via N-terminal hydrophobic stretches. Carboxy terminal PHB (SPFH) and coiled-coildomains are exposed to the intermembrane space (IMS). IM = inner membrane.

Prohibitins make a puzzle that is really difficult to understand. They are involved in several different processes in at least three distinct compartments, forming complexes and reacting with many various proteins. Situation is complicated by changes that occur from organism to organism and cell to cell.

Why so many functions? PHBs are proteins, designed to react and interact with others and they are definitely used in processing some signals. For example regulating the signal of estrogen receptor takes part by stabilizing complexes by physical interaction between PHBs and their binding partners (He et al., 2008). They are definitely preadapted to interact with other proteins, and probably only small changes in their structure allow them to gain a new function (Jain et al., 2010, Emerson et al., 2010) seemingly without loss of the original one. We are gradually getting to know with which proteins PHBs do react. What we actually do not understand now is the specificity of their interaction with other proteins and how it is controlled.

PHBs are in the center of many important processes. Localized in the plasma membrane, they convey the signal from outer environment of the cell and facilitate interaction with other cells. As transcription cofactors, they contribute to decisions what protein and when to produce and it seems that they also play some role in cancerogenesis. As chaperons and scaffold proteins they are present also on another level of regulation of protein synthesis and morphogenesis, linkage with m-AAA proteases even give them the power to degrade some proteins, or proteolytically activate others. Prohibitins are one of those proteins that control the power plant of the cell - the mitochondrion -

and thus the fate of the whole cell and potentially the whole organism (neurodegenerative diseases).
Yet none of this function can be confined solely to prohibitins themselves, so we are in the middle of the really complicated net inside the cell looking for some ends from which we can start to solving this brain teaser.
Prohibitins definitely did not reveal all their secrets and every new discovery so far brings more questions and only few answers. But the pieces of the PHBs mosaic are coming together as more and more information is gathered. We are now aware of many processes in which PHBs play role. On the protein level several binding partners were already identified and many other proteins affected by PHBs are known. We tried to contribute to this mosaic with describing the situation in procyclic *Trypanosoma brucei*. I do believe that in the end we will be able to put all the pieces of this mosaic together and decipher all the PHBs secrets.

8. LITERATURE

Almeida, R., Gilmartin, B.J., McCann, S.H., Norrish, A., Ivens, A.C., et al., 2004. Expression profiling of the *Leishmania* life cycle: cDNA arrays identify developmentally regulated genes present but not annotated in the genome. Mol. Biochem. Parasitol. 136, 87-100

Artal-Sanz, M., Tavernarakis, N., 2009a. Prohibitin couples diapause signalling to mitochondrial metabolism during ageing in C. elegans. Nature. 461(7265), 793-7

Artal-Sanz, M., Tavernarakis, N., 2009b. Prohibitin and mitochondrial biology. Trends. Endocrinol. Metab. 20(8),394-401

Broadhead, R., Dawe, H.R., Farr, H., Griffiths, S., Hart, S.R., Portman, N., Shaw, M.K., Ginger, M.L., Gaskell, P.G., McKean, P.G., Gull, K., 2006. Flagellar motility is required for the viability of the bloodstream trypanosome. Nature 440(7081),224-7

Cunningham, A.C., 2002. Parasitic adaptive mechanisms in infection by leishmania. Exp. Mol. Pathol. 72(2),132-41

Emerson, V., Holkotte, D., Pfeiffer, T., Wang, I.H., Schnolzer, M., Kempf, T., Bosch, V. 2010. Identification of the cellular prohibitin 1/Prohibitin 2 heterodimer as an interaction partner of the C-terminal cytoplasmic domain of the HIV-1 glycoprotein. J. Virol. 84,3,1355-1365

He, B., Fenq, Q., Mukherjee, A., Lonard, D.M., DeMayo, F.J., Katzenellenbogen, B.S., Lydon, J.P., O'Malley, B.W., 2008. A repressive role for prohibitin in estrogen signaling.
Mol. Endocrinol. 22, 344–360

Heron-Milhavet, L., Mamaeva, D., Rochat, A., Lamb, N.L., Fernandez, A., 2008. Akt2 is implicated in skeletal muscle differentiation and specifically binds Prohibitin2/REA. J. Cell. Physiol. 214,158–165

Jain, R., Ghoshal, A., Mandal, C., Shaha, C., 2010. Leishmania cell surface prohibiti: role in host-parasite interaction. Cell. Microbiol. 12(4), 432-452

Kim, J.W., Akiyama, M., Park, J.H., Lin, M.L., Shimo, A., Ueki, T., Daigo, Y., Tsunoda, T., Nishidate, T., Nakamura, Y., Katagiri, T. 2009. Activation of an estrogen/estrogen receptor signaling by BIG3 through its inhibitory effect on nuclear transport of PHB2/REA in breast cancer. Cancer Sci. 100,8, 1468-1478

Matthews, K.R., 2005. The developmental cell biology of *Trypanosoma brucei*. J. Cell. Sci. 118,283-290

Merkwirth, C., Dargazanli, S., Tatsuta, T., Geimer, S., Lower, B., Wunderlich, F.T., Kleist-Retzow, J.C., Waisman, A., Westermann, B., Langer, T., 2008. Prohibitins control cell proliferation and apoptosis by regulation OPA1-dependent cristae morphogenesis in mitochondria. Gen. Dev. 22, 476-488

Merkwirth, C., Langer, T., 2009. Prohibitin function within mitochondria: essential roles for cell proliferation and cristae morphogenesis. Biochim. Biophys. Acta. 1793(1), 27-32

Plechota, J., Kolodzijczak, M., Juszczak, I., Sakamoto, W., Janska, H., 2010. Identification and characterization of high molecular weight complexes formed by matrix AAA proteases and prohibitins in mitochondria of Arabidopsis thaliana. J. Biol. Chem. 17, 12512-12521

Rajalingam, K., Rudel, T,. 2005. Ras-Raf signaling needs prohibitin. Cell Cycle 4,1503–1505

Sharma, A., Aadri, A., 2004. Vi polysaccharide of Salmonella typhi targets the prohibitin family of molecules in intestinal epithelial cells and suppresses early inflammatory responses. Proc. Natl. Acad. Sci. U. S. A. 101,17492–17497

Sikora, J., Towpik, J., Graczyk, D., Kistowski, M., Rubel, T., Poznanski, J., Langridge, J., Hughes, C., Dadlez, M., Boguta, M. 2009. Yeast prion [PSI+] lowers the lewels of mitochondrial prohibitins. Biochem. Biophys. Act. 1793, 1703-1709

Steglich, G., Neupert, W., Langer, T., 1999. Prohibitins regulate membrane protein degradation by the m-AAA protease in mitochondria, Mol. Cell. Biol. 19, 3435–3442.

Terashima, M., Kim, K.M., Adachi, T., Nielsen, P.J., Reth, M., Kohler, G., Lamers, M.C., 1994. The IgM antigen receptor of B lymphocytes is associated with prohibitin and a prohibitin-related protein. EMBO J. 13,3782–3792

Vertommen, D., Roy, J.V., Szikora, J.P., Rider, M.H., Michels, P.A.M., Opperdoes, F.R., 2007. Differential expression of glycosomal and mitochondrial proteins in two major life-cycle stages of *Trypanosoma brucei*. MBP. 158, 189-201

Yao, C., Li, L., Donelson, J.E., Wilson, M.E., 2010. Proteomic examination of *Leishmania chagasi* plasma membrane proteins: contrast between avirulent and virulent (metacyclic) parasite forms. Prot. Clin. Appl. 4,4-16