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Perspective

# Small molecule inhibition of protein depalmitoylation as a new approach towards downregulation of oncogenic Ras signalling

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ABSTRACT

The H- and N-Ras GTPases are prominent examples of proteins, whose localizations and signalling capacities are regulated by reversible palmitoylations and depalmitoylations. Recently, the novel small molecule inhibitor palmostatin B has been described to inhibit Ras depalmitoylation and to revert the phenotype of oncogenic HRasG12V transformed cells. This demonstrates that palmostatin B is a tool to investigate the biochemical effects of the inhibition of cellular Ras depalmitoylation on Ras signalling, which is relevant for oncology. Furthermore, it is to be expected that many proteins, of which the signalling capacities depend on reversible palmitoylation, will be discovered in the near future. This stresses the urgent need for further development of small molecule inhibitors of palmitoylation and depalmitoylation in order to study their functions in cellular signalling.



## 1. Introduction

### 1.1. Ras signalling

Ras GTPases are molecular switches that are, among others, activated by extracellular signals via protein tyrosine kinase receptors, such as, for example, the endothelial growth factor (EGF) receptor. Tyrosine phosphorylation in the cytosolic domain leads to binding of adaptor proteins, such as Grb2 and guanine exchange factor (GEF) SOS that activates Ras GTPases. Activation results in guanosine-triphosphate (GTP) bound Ras, which in turn activates multiple downstream effectors and thus triggers changes in cellular behaviour.[1–3] The best characterized pathway is the mitogen activated protein kinases (MAPK) pathway, which involves Raf-1 kinase, MEK1/2 and ERK1/2. Activated Ras can also bind and activate phosphoinositide-3-kinase (PI3K), which catalyses the formation of the second messenger phosphatidylinositol-3,4,5-triphosphate.[4] Another Ras effector is phospholipase Cε (PLCε) that links Ras activation to protein kinase C (PKC) activation and calcium mobilization.[5] Under normal conditions, Ras activation results from specific extracellular signals and is transient. However, Ras GTPases are mutationally activated in several types of cancer.[6,7] Mutationally activated Ras activates downstream effectors independently from extracellular signals and thus contributes to oncogenesis. Therefore, enzymes that regulate Ras activity gained attention as potential drug targets for cancer therapy.[8]

Although the four Ras isoforms, N-Ras, H-Ras, K-Ras4A and K-Ras4B, are encoded by different genes, the Ras domains that are required for activation are nearly identical in the different isoforms.[9] The differences reside in the C-terminal region referred to as hypervariable region (HVR), which is modified by lipid posttranslational modifications. H-Ras is modified by two cysteine palmitoylations and one cysteine farnesylation, whereas N-Ras and K-Ras(A) are modified by one cysteine palmitoylation and one cysteine farnesylation. In contrast, K-Ras(B) is not palmitoylated. Reversible palmitoylations of H- and N-Ras GTPases control their membrane attachment and specific localization on the plasma membrane and the Golgi.[10–13] Proper steady state localization requires a dynamic cycle of palmitoylation on the Golgi, which redirects Ras to the plasma membrane, and ubiquitous depalmitoylation to counteract spontaneous non specific distribution over cellular endomembranes (Fig. 1).[12] Disruption of this dynamic cycle results in a reduction of Ras localization on the Golgi and the plasma membrane, due to random redistribution to endomembranes. This indicates that inhibitors of palmitoylation as well as depalmitoylation alter the steady state localization of Ras GTPases and thus Ras signalling.

### 1.2. Inhibition of palmitoylation

Inhibition of palmitoylation prevents membrane attachment of Ras GTPases (Fig. 1), which results in inhibition of oncogenic Ras signalling. The enzymes that are responsible for Ras palmitoylation are referred to as palmitoyl acyltransferases (PATs).[14] The best know inhibitor of protein palmitoylation is 2-bromopalmitate (2BP),[12,15] which contains, however, a reactive functionality that

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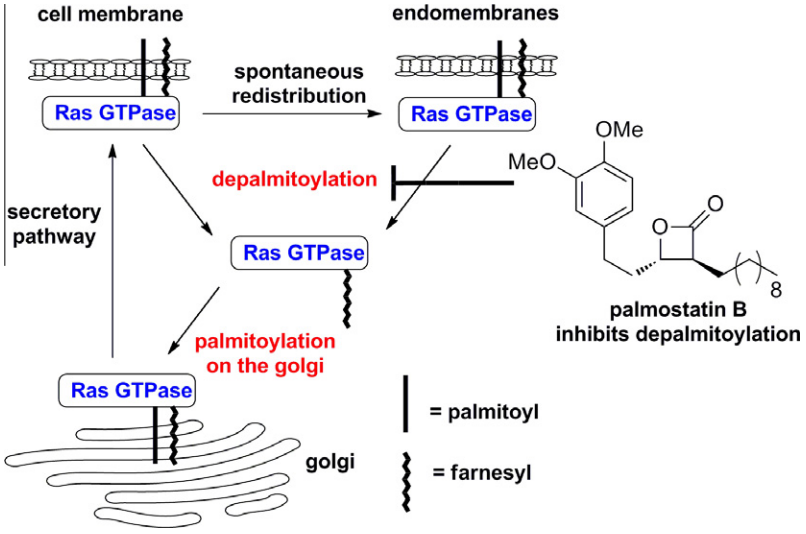


**Figure 1.** The specific subcellar localization of Ras GTPases is maintained by a dynamic cycle of ubiquitous depalmitoylation and palmitoylation on the Golgi system. Depalmitoylation followed by palmitoylation on the Golgi redirects Ras GTPases to the plasma membrane through the secretory pathway. Inhibition of palmitoylation or depalmitoylation provides an approach to disturb Ras localization and to inhibit local Ras activity and downstream signalling. Interfering with depalmitoylation, by palmostatin B, leads to random distribution of Ras GTPases over endomembranes and thereby uncouples spatial regulated signalling pathways.

limits its selectivity. Ducker et al. have identified five different small molecules without reactive functionalities that inhibit protein palmitoylation in vitro.[16] These compounds inhibit plasma membrane binding of GFP-labelled H-Ras constructs and also Ras mediated signalling via the MAPK pathway. Development of small molecule inhibitors of Ras palmitoylation is therefore considered as a viable strategy to downregulate oncogenic Ras signalling.

### 1.3. Inhibition of depalmitoylation

In contrast to the palmitoylating enzymes, of which several enzyme subtypes have been characterized, only one cytosolic depalmitoylating enzyme has been described. The depalmitoylating enzyme acyl protein thioesterase 1 (APT1) was initially described as a lysophospholipase.[17] It has been shown that palmitoylated proteins are the preferred substrates for APT1 in vitro.[18] In addition, overexpression of this thioesterase in cellular studies demonstrated up-regulation of depalmitoylation of heterotrimeric G protein α subunits and H-Ras.[18] Another study demonstrated that APT1 is involved in depalmitoylation of eNOS.[19] In contrast, APT1 does not depalmitoylate caveolin.[19] This suggests that different depalmitoylating enzymes exist, which have not been discovered yet, or that other, maybe non-enzymatic processes, are involved in depalmitoylation.

Development of APT1 inhibitors gained little attention in oncology, because it has been expected that inhibition of depalmitoylation increases the palmitoylated state of the Ras GTPase and thus the membrane attachment, which would facilitate oncogenic signalling. However, the discovery that dynamic palmitoylation and depalmitoylation is required to maintain proper Ras localization indicates that inhibition of Ras depalmitoylation will disturb Ras localization und thus Ras signalling.[12,13] This was the rationale to develop the recently described Ras depalmitoylation inhibitor palmostatin B in order to downregulate oncogenic Ras signalling.[15]

## 2. Palmostatin B

### 2.1. Palmostatin B inhibits acyl protein thioesterase 1

Palmostatin B was developed using a knowledge based strategy, denoted protein structure similarity clustering (PSSC).[20–22] In this strategy proteins are assigned to clusters based on similarity in their three dimensional structure in their ligand sensing cores. Using this approach structural similarity was observed between the enzymes APT1 and gastric lipase (Fig. 2). The gastric lipase inhibitor tetrahydrolipstatin was employed to design a focused compound collection with a β-lactone core structure (Fig. 2). This compound collection was screened for inhibition of the enzyme APT1 and provided the inhibitor palmostatin B as the most potent compound ($IC_{50}$ 670 nM). Palmostatin B inactivates APT1 by reversible covalent modification of the enzyme active site. The enzyme is acylated by palmostatin B through nucleophilic opening of the β-lactone electrophile, followed by regeneration of the active enzyme by hydrolysis of the acylated enzyme. Furthermore, a direct interaction between APT1 and palmostatin B in cells was demonstrated. Palmostatin B inhibits APT1 selectively compared with the intracellular phospholipases A1, A2, Cβ and D. Thus, palmostatin B is a selective small molecule inhibitor that behaves as slowly converted substrate and thus temporarily inhibits the APT1 enzyme activity.[15]

### 2.2. Palmostatin B inhibits Ras depalmitoylation

Normally, H- and N-Ras are mainly bound to the plasma membrane and the Golgi.[12] This specific distribution was lost upon treatment with palmostatin B, which is in line with the model in Figure 1.[15] According to this model depalmitoylation serves as a reset mechanism against random distribution over endomembranes.[12] Consequently, inhibition of depalmitoylation blocks the transport of H- and N-Ras back to the Golgi. It has been shown that Palmostatin B treatment inhibited depalmitoylation of N-Ras in cell-based studies.[15] The same effect was found after siRNA mediated downregulation of APT1 expression.[15] The kinetics of localized cellular Ras palmitoylation and depalmitoylation was studied using semisynthetic Ras GTPases (Fig. 3A).[15] The semisynthetic Ras GTPases were obtained via a combination or organic synthesis and protein expression. Ras GTPases with truncated C-termini were expressed and the C-terminal peptides, with the appropriate posttranslational modifications, were synthesized by organic chemistry methods. The C-terminal peptides were linked covalently to the truncated Ras GTPases to provide functional Ras GTPases that were fluorescently labelled.[23–25] These semisynthetic Ras GTPases were micro-injected into livings cells and accumulation on the Golgi was measured over

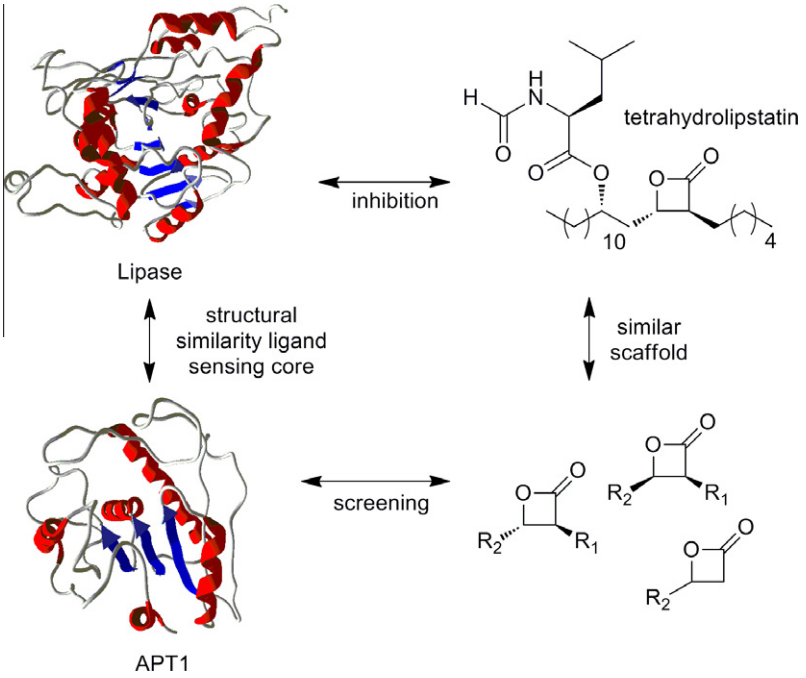


**Figure 2.** A knowledge based approach was used for discovery of palmostatin B as APT1 inhibitor. Protein structure similarity clustering (PSSC) revealed structural similarity between lipase and APT1 in their ligand sensing cores. The lipase inhibitor tetrahydroplipstatin was used for synthesis of a focused compound collection that was screened for inhibition of APT1.

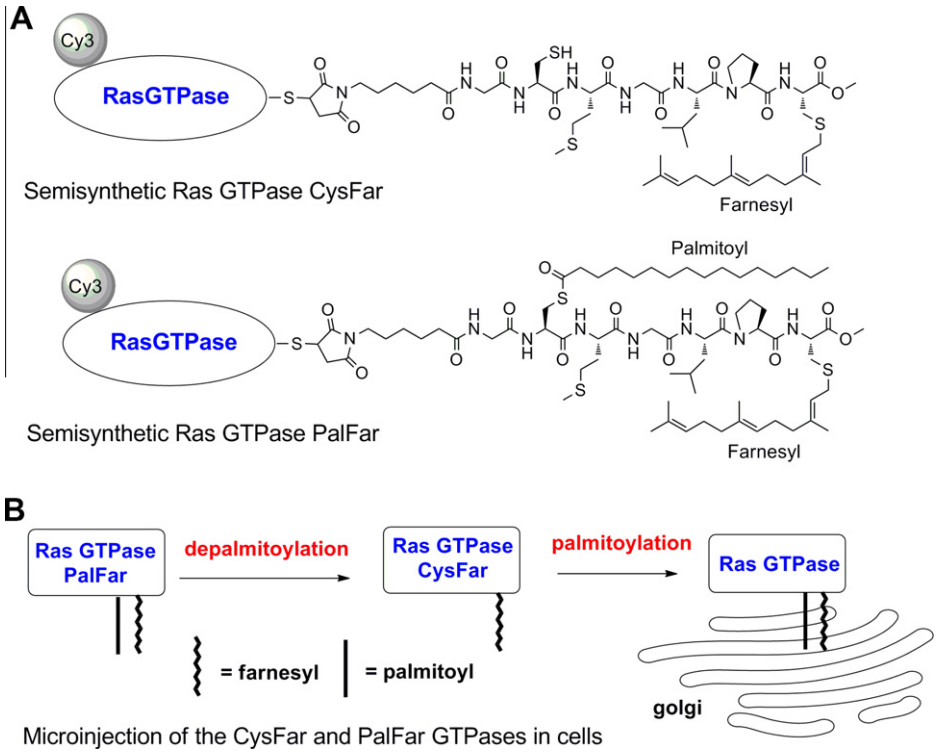


**Figure 3.** The local palmitoylation rate on the Golgi can be studied using semisynthetic Ras proteins. (A) Semisynthetic Ras GTPases are synthesized by expression of truncated Ras GTPases that lack their C-termini. The Ras C-termini, with their appropriate posttranslational modifications, are synthesized using organic chemistry methods. The synthetic C-termini are equipped with a maleïmide functionality that is linked covalently to the truncated Ras GTPases to provide a functional Ras GTPase. The semisynthetic Ras GTPases are labelled with a fluorophore that allows detection in living cells. (B) The semisynthetic Ras GTPases are micro-injected in living cells and the kinetics of the fluorescence increase on the Golgi is measured. Upon microinjection of the semisynthetic Ras GTPase CysFar the fluorescence increase on the Golgi reflects the rate of trapping of the Ras GTPase on the Golgi by palmitoylation. Upon microinjection of the semisynthetic Ras GTPase PalFar the fluorescence increase on the Golgi reflects the rate of trapping of the Ras GTPase on the Golgi by depalmitoylation and subsequent palmitoylation. Palmostatin B inhibits the depalmitoylation of the semisynthetic Ras GTPase PalFar and not the palmitoylation.[15]

time. The accumulation of the semisynthetic Ras GTPase CysFar on the Golgi is a reflection of the palmitoylation rate of the Ras GTPase on the Golgi, whereas the accumulation of the Ras GTPase PalFar on the Golgi is a reflection of the rate of cellular depalmitoylation followed by palmitoylation on the Golgi (Fig. 3B).[12,15] Palmostatin B inhibited the Golgi accumulation of the Ras GTPase PalFar and not CysFar. This demonstrates that palmostatin B inhibits Ras depalmitoylation and not palmitoylation in living cells.

Interestingly, palmostatin B had a pronounced effect on the compartment specific activation of H-Ras. Normally, growth factor stimulation leads to rapid and transient activation of H-Ras on the plasma membrane and slow but sustained activation on the Golgi.[12] Short term treatment with palmostatin B inhibited H-Ras activation on the Golgi, whereas H-Ras activation on the plasma membrane was not disturbed.[15] Thus, palmostatin B is a tool to uncouple Ras activity on both compartments and to study the influence on the Ras signalling output. Finally, it was demonstrated that palmostatin B causes phenotypic reversion of oncogenic Ras transformed cells. Oncogenic Ras transformed MDCK-f3 cells show a long and spindle like phenotype and grow in multiple layers with reduced cell–cell contacts. Palmostatin B caused reversion to the round phenotype that is characteristic for untransformed MDCK cells.

### 2.3. Palmostatin B selectivity

Selectivity for different enzyme subtypes is, in general, a major concern for small molecule inhibitors. Palmostatin B shows selective inhibition of APT1 compared to cellular phospholipases, such as phospholipases A1, A2, Cβ and D.[15] The fact that palmostatin B behaves as slowly converted substrate for APT1 might cause this selectivity. The APT1 inhibitory potency is determined by a relatively fast covalent inhibition rate constant as well as a relatively slow reactivation rate constant. Palmostatin B might fail to inhibit the other enzymes by either a relatively slow inactivation rate or a relatively quick reactivation rate. Nevertheless, for further efforts in drug discovery, it would be important to evaluate the selectivity towards an even broader group of enzymes. Enzymes to be included in this group are diverse members of the broad family of esterases. Furthermore, enzymes with related sequences such as acyl protein thioesterase 2 (APT2) might be inhibited by palmostatin B. Additionally, protein structure similarity clustering (PSSC)[21,22] could give indications on the selection of targets to evaluate the palmostatin B selectivity. However, it should be noted that selective inhibition is commonly found for targets with functional, structural or sequence similarity.

### 2.4. Selectivity in cellular Ras depalmitoylation

Two studies describe the selectivity of cellular Ras depalmitoylation, which is for a significant part mediated by APT1.[15] One study describes that no selectivity is observed in cellular depalmitoylation of semisynthetic Ras GTPases with structurally diverse C-termini.[26] Another study describes pulse-chase experiments, which revealed that oncogenic H-Ras isoforms are quicker depalmitoylated than wild type H-Ras.[27] These differences were attributed to differences in the conformations of GTP and guanosine-diphosphate (GDP) bound Ras. The half life for palmitate removal from GTP bound Ras was estimated to be less than 10 min, which is about 20 times faster than for GDP bound Ras. This raises the idea that cellular depalmitoylation does not depend on the structure of the Ras C-terminus, but on the structure of Ras itself. The limited knowledge of cellular depalmitoylating enzymes and their substrate selectivity calls for further exploration of this enzyme class.

### 2.5. Palmostatin B inhibits Ras activation on the Golgi

Interestingly, Ras signalling does not only originate from the plasma membrane but also from other cellular compartments such as the Golgi.[28] Different downstream effects have been described for compartment specific Ras activity and compartment specific signalling has been demonstrated for individual Ras isoforms.[28] H- and N-Ras activation on the PM is rapid and transient, whereas activation of Ras on the Golgi is delayed and sustained.[12,29] Furthermore, Ras activation on different compartments is cell-type and signal dependent. It has been shown that Ras can be activated on the Golgi independently from activation on the plasma membrane in Jurkat cells upon T-cell receptor stimulation.[30] This activation of Ras on the Golgi involves the GEF RasGRP1, which is regulated by calcium and diacylglycerol.[30] This suggests a model in which Ras on the Golgi is activated via water soluble second messengers and GEFs as reviewed by Quatela and Philips.[31] In contrast to this established model, endothelial growth factor (EGF) mediated activation of Ras on the Golgi seems to involve Ras depalmitoylation, because short term palmostatin B treatment, which inhibits Ras depalmitoylation, inhibits EGF mediated activation of H-Ras on the Golgi, whereas H-Ras activation on the plasma membrane is maintained.[15] This argues for a model in which Ras is activated on the plasma membrane and is then trafficked to the Golgi by depalmitoylation in its GTP bound form. It should, however, be noted that palmostatin B might influence other signalling mechanisms that are independent from Ras activation on the plasma membrane. Which model applies for specific signals and if the second model is correct for EGF mediated signal transduction remains to be elucidated.

## 3. Lipid modifications in signal transduction

### 3.1. Ras signalling through membrane microdomains

It becomes increasingly clear that lateral segregation of Ras GTPases into microdomains on the plasma membrane plays an important role in Ras mediated signal transduction.[32] It has been demonstrated that both H-Ras palmitoylation sites serve distinct roles in membrane attachment and localization to microdomains.[33] Furthermore, microdomains with GDP loaded H-, N- and K-Ras are not able to activate the MAPK pathway by Raf activation, whereas microdomains with GTP loaded Ras are capable of Raf activation.[34] These microdomains are thought to facilitate signalling by concentrating the Ras GTPases to specific locations.[35–37] Recently, it has been demonstrated that the formation of EGF receptor microdomains require receptor tyrosine kinase activity and the formation of phosphatidic acid by phospholipase D2 (PLD2).[38] Inhibition of the PLD2 activity inhibited activation of the MAPK signalling pathway. These studies show that small molecule inhibitors of lipid modifying enzymes might interfere with multiple processes that are crucial for Ras mediated signalling.

### 3.2. Depalmitoylation of eNOS and Gα

Depalmitoylation is not only important for Ras mediated signal transduction and oncology, but also for other signalling pathways and disease models. For example, Siegel et al. demonstrated that APT1 is involved in dentritic spine morphogenesis.[39] Both palmostatin B (in this study denoted FD196) mediated inhibition of APT1 activity and shRNA mediated downregulation of APT1 expression reduced dendritic spine volume. Interestingly, APT1 expression is inhibited by microRNA 138. $G_{\alpha 13}$ palmitoylation is required for membrane localization and Rho-dependent signalling,[40] which suggests that inhibition of depalmitoylation increases $G_{\alpha 13}$

membrane attachment and thus increases Rho-signalling. The involvement of APT1 in spine morphogenesis links APT1 inhibition to effects on the nervous system, which might be either beneficial or detrimental for APT1 directed drug discovery. Furthermore, APT1 is also involved in eNOS depalmitoylation, which is linked to vascular tone and platelet aggregation. Interestingly, APT1 catalyzed depalmitoylation of eNOS is potentiated by $Ca^{2+}$-calmodulin, which is an allosteric modulator of eNOS.[19] In contrast, APT1 catalyzed depalmitoylation of $G_{\alpha s}$ is unaffected by $Ca^{2+}$-calmodulin, which demonstrates that depalmitoylation is not only directed by depalmitoylating enzymes, such as APT1, but also by allosteric regulators of palmitoylated proteins.

### 3.3. Depalmitoylation and the palmitoylome

Inhibitors of depalmitoylation, such as palmostatin B, will gain importance in the near future, because a recently developed methodology enables the identification of all palmitoylated proteins in cell-lysates, which is referred to as the palmitoylome.[41] This methodology employs the small molecule 17-octadecynoic acid that serves as a bioorthogonal probe for labelling of palmitoylated proteins in human cells.[41] The power of this methodology is illustrated by pulse-chase studies on Lck S-deacylation. This study reveals that the broad spectrum serine hydrolase inhibitor methylarachidonylfluorophosphonate (MAFP) reduces Lck depalmitoylation, which indicates the involvement of thioesterases like for example APT1.[42] Another example of the power of bioorthogonal labelling of palmitoylated proteins is the discovery that S-palmitoylation of the interferon induced transmembrane protein 3 (IFITM3) controls its clustering in membrane compartments and thus its antiviral activity.[43] These studies demonstrate that bioorthogonal labelling of palmitoylated proteins in combination with small molecule inhibitors of depalmitoylation enable the identification of proteins, whose functions are regulated by palmitoylation and depalmitoylation. It is to be expected that many of such proteins will be discovered in the near future.

## 4. Conclusion

Palmostatin B is a small molecule that inhibits Ras depalmitoylation, which has a profound effect on Ras localization and signalling.[15] Short term inhibition prevents Ras activation on the Golgi, which enables studies on compartment specific signalling. Alternatively, long term inhibition of Ras depalmitoylation disturbs its precise steady state localization and thus inhibits Ras signalling, which is reflected by the phenotypic backtransformation of oncogenic Ras transformed MDCK cells. This demonstrates that palmostatin B is a tools to study Ras depalmitoylation in cell-based studies. Furthermore, it opens a new and unexpected avenue towards further development of conceptually new therapeutic approaches in oncology that target depalmitoylation. Moreover, it is expected that many proteins that are regulated by reversible palmitoylation and many palmitoyl transferases and thioesterases will be identified in the near future, which will increase the importance of thioesterase inhibitors such as palmostatin B.

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## Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2010.11.025. These data include MOL files and InChiKeys of the most important compounds described in this article.

## References and notes

1. Downward, J. *Nat. Rev. Cancer* **2003**, *3*, 11.
2. Bourne, H. R.; Sanders, D. A.; McCormick, F. *Nature* **1990**, *348*, 125.
3. Schubbert, S.; Shannon, K.; Bollag, G. *Nat. Rev. Cancer* **2007**, *7*, 295.
4. Rodriguez-Viciana, P.; Warne, P. H.; Dhand, R.; Vanhaesebroeck, B.; Gout, I.; Fry, M. J.; Waterfield, M. D.; Downward, J. *Nature* **1994**, *370*, 527.
5. Kelley, G. G.; Reks, S. E.; Ondrako, J. M.; Smrcka, A. V. *EMBO J.* **2001**, *20*, 743.
6. Reddy, E. P.; Reynolds, R. K.; Santos, E.; Barbacid, M. *Nature* **1982**, *300*, 149.
7. Hall, A.; Marshall, C. J.; Spurr, N. K.; Weiss, R. A. *Nature* **1983**, *303*, 396.
8. Saxena, N.; Lahiri, S. S.; Hambarde, S.; Tripathi, R. P. *Cancer Invest.* **2008**, *26*, 948.
9. Eisenberg, S.; Henis, Y. I. *Cell. Signalling* **2008**, *20*, 31.
10. Linder, M. E.; Deschenes, R. J. *Nat. Rev. Mol. Cell. Biol.* **2007**, *8*, 74.
11. Greaves, J.; Chamberlain, L. H. *J. Cell. Biol.* **2007**, *176*, 249.
12. Rocks, O.; Peyker, A.; Kahms, M.; Verveer, P. J.; Koerner, C.; Lumbierres, M.; Kuhlmann, J.; Waldmann, H.; Wittinghofer, A.; Bastiaens, P. I. H. *Science* **2005**, *307*, 1746.
13. Goodwin, J. S.; Drake, K. R.; Rogers, C.; Wright, L.; Lippincott-Schwartz, J.; Philips, M. R.; Kenworthy, A. K. *J. Cell Biol.* **2005**, *170*, 261.
14. Mitchell, D. A.; Vasudevan, A.; Linder, M. E.; Deschenes, R. J. *J. Lipid Res.* **2006**, *47*, 1118.
15. Dekker, F. J.; Rocks, O.; Vartak, N.; Menninger, S.; Hedberg, C.; Balamurugan, R.; Wetzel, S.; Renner, S.; Gerauer, M.; Schölermann, B.; Rusch, M.; Kramer, J. W.; Rauh, D.; Coates, G. W.; Brunsveld, L.; Bastiaens, P. I.; Waldmann, H. *Nat. Chem. Biol.* **2010**, *6*, 449.
16. Ducker, C. E.; Griffel, L. K.; Smith, R. A.; Keller, S. N.; Zhuang, Y.; Xia, Z.; Diller, J. D.; Smith, C. D. *Mol. Cancer Ther.* **2006**, *5*, 1647.
17. Sugimoto, H.; Hayashi, H.; Yamashita, S. *J. Biol. Chem.* **1996**, *271*, 7705.
18. Duncan, J. A.; Gilman, A. G. *J. Biol. Chem.* **1998**, *273*, 15830.
19. Yeh, D. C.; Duncan, J. A.; Yamashita, S.; Michel, T. *J. Biol. Chem.* **1999**, *274*, 33148.
20. Balamurugan, R.; Dekker, F. J.; Waldmann, H. *Mol. Biosyst.* **2005**, *1*, 36.
21. Koch, M. A.; Wittenberg, L. O.; Basu, S.; Jeyaraj, D. A.; Gourzoulidou, E.; Reinecke, K.; Odermatt, A.; Waldmann, H. *Proc. Natl. Acad. Sci. USA* **2004**, *101*, 16721.
22. Dekker, F. J.; Koch, M. A.; Waldmann, H. *Curr. Opin. Chem. Biol.* **2005**, *9*, 232.
23. Reents, R.; Wagner, M.; Kuhlmann, J.; Waldmann, H. *Angew. Chem., Int. Ed.* **2004**, *43*, 2711.
24. Brunsveld, L.; Kuhlmann, J.; Waldmann, H. *Methods* **2006**, *40*, 151.
25. Brunsveld, L.; Kuhlmann, J.; Alexandrov, K.; Wittinghofer, A.; Goody, R. S.; Waldmann, H. *Angew. Chem., Int. Ed.* **2006**, *45*, 6622.
26. Rocks, O.; Gerauer, M.; Vartak, N.; Koch, S.; Huang, Z. P.; Pechlivanis, M.; Kuhlmann, J.; Brunsveld, L.; Chandra, A.; Ellinger, B.; Waldmann, H.; Bastiaens, P. I. *Cell* **2010**, *141*, 458.
27. Baker, T. L.; Zheng, H.; Walker, J.; Coloff, J. L.; Buss, J. E. *J. Biol. Chem.* **2003**, *278*, 19292.
28. Omerovic, J.; Laude, A. J.; Prior, I. A. *Cell. Mol. Life Sci.* **2007**, *64*, 2575.
29. Chiu, V. K.; Bivona, T.; Hach, A.; Sajous, J. B.; Silletti, J.; Wiener, H.; Johnson, R. L.; Cox, A. D.; Philips, M. R. *Nat. Cell Biol.* **2002**, *4*, 343.
30. Bivona, T. G.; Pérez De Castro, I.; Ahearn, I. M.; Grana, T. M.; Chiu, V. K.; Lockyer, P. J.; Cullen, P. J.; Pellicer, A.; Cox, A. D.; Philips, M. R. *Nature* **2003**, *424*, 694.
31. Quatela, S. E.; Philips, M. R. *Curr. Opin. Cell Biol.* **2006**, *18*, 162.
32. Henis, Y. I.; Hancock, J. F.; Prior, I. A. *Mol. Membr. Biol.* **2009**, *26*, 80.
33. Roy, S.; Plowman, S.; Rotblat, B.; Prior, I. A.; Muncke, C.; Grainger, S.; Parton, R. G.; Henis, Y. I.; Kloog, Y.; Hancock, J. F. *Mol. Cell. Biol.* **2005**, *25*, 6722.
34. Inder, K.; Harding, A.; Plowman, S. J.; Philips, M. R.; Parton, R. G.; Hancock, J. F. *Mol. Biol. Cell.* **2008**, *19*, 4776.
35. Harding, A. S.; Hancock, J. F. *Trends Cell Biol.* **2008**, *18*, 364.
36. Harding, A.; Hancock, J. F. *Cell Cycle* **2008**, *7*, 127.
37. Tian, T.; Harding, A.; Inder, K.; Plowman, S.; Parton, R. G.; Hancock, J. F. *Nat. Cell Biol.* **2007**, *9*, 905.
38. Ariotti, N.; Liang, H.; Xu, Y.; Zhang, Y.; Yonekubo, Y.; Inder, K.; Du, G.; Parton, R. G.; Hancock, J. F.; Plowman, S. J. *Mol. Cell. Biol.* **2010**, *30*, 3795.
39. Siegel, G.; Obernosterer, G.; Fiore, R.; Oehmen, M.; Bicker, S.; Christensen, M.; Khudayberdiev, S.; Leuschner, P. F.; Busch, C. J.; Kane, C.; Hübel, K.; Dekker, F.; Hedberg, C.; Rengarajan, B.; Drepper, C.; Waldmann, H.; Kauppinen, S.; Greenberg, M. E.; Draguhn, A.; Rehmsmeier, M.; Martinez, J.; Schratt, G. M. *Nat. Cell Biol.* **2009**, *11*, 705.
40. Bhattacharyya, R.; Wedegaertner, P. B. *J. Biol. Chem.* **2000**, *275*, 14992.
41. Martin, B. R.; Cravatt, B. F. *Nat. Methods* **2009**, *6*, 135.
42. Zhang, M. M.; Tsou, L. K.; Charron, G.; Raghavan, A. S.; Hang, H. C. *Proc. Natl. Acad. Sci. USA* **2010**, *107*, 8627.
43. Yount, J. S.; Moltedo, B.; Yang, Y. Y.; Charron, G.; Moran, T. M.; López, C. B.; Hang, H. C. *Nat. Chem. Biol.* **2010**, *6*, 610.