

Different Enzymatic Processing of γ -Phosphoramidate and γ -Phosphoester-Modified ATP Analogues

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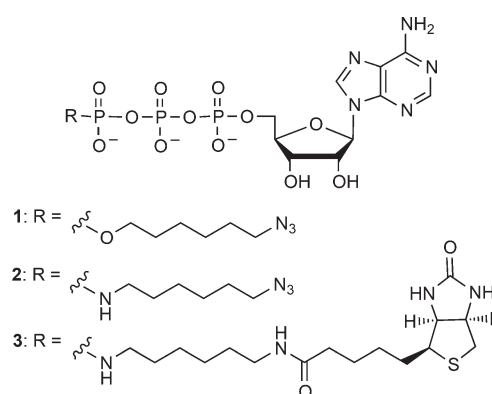
Monitoring the activity of ATP-consuming enzymes provides the basis for elucidating their modes of action and regulation. Although a number of ATP analogues have been developed for this, their scope is restricted because of the limited acceptance by respective enzymes. In order to clarify which kind of phosphate-modified ATP analogues are accepted by the α - β -phosphoanhydride-cleaving ubiquitin-activating enzyme 1 (UBA1) and the β - γ -phosphoanhydride-cleaving focal adhesion kinase (FAK), we tested phosphoramidate- and phosphoester-modified ATP analogues. UBA1 and FAK were able to convert phosphoramidate-modified ATP analogues, even with a bulky modification like biotin. In contrast, a phosphoester-modified analogue was poorly accepted. These results demonstrate that minor variations in the design of ATP analogues for monitoring ATP utilization have a significant impact on enzymatic acceptance.

Introduction

Adenosine triphosphate (ATP) is the most common cosubstrate in otherwise endergonic enzymatic reactions chemical reactions and is therefore involved in many intracellular processes.^[1] Thus, numerous ATP-cleaving enzymes of distinct classes exist. Important examples of ATP-consuming enzymes are ion pumps (e.g., Ca^{2+} -ATPases and the Na^+/K^+ -ATPases),^[2] motor proteins (e.g., kinesins,^[3] dyneins,^[4] myosins^[5]), acyl-transferring enzymes (e.g., acyl-CoA synthetases,^[6] aminoacyl-tRNA-synthetases,^[7] and ubiquitin-activating enzymes^[8]), RNA polymerases,^[9] and kinases.^[10] Therefore, studying the turnover of ATP allows investigating a variety of cellular processes. Classical strategies make use of radioactively labeled ATP^[11] or measure the released phosphate or pyrophosphate (PP) by colorimetric detection.^[12] Another common method is the chemical modification of ATP at the γ -phosphate. The majority of such analogues are phosphoramidate-modified ATP analogues,^[13] although they are prone to hydrolysis under acidic conditions.^[14]

ATP analogues modified with a phosphothioester,^[15] phosphoester^[14,16] or phosphonate^[14] have also been reported.

In this study, two ATP-cleaving enzymes from different enzyme classes, ubiquitin-activating enzyme 1 (UBA1) and focal adhesion kinase (FAK), were compared for their propensity of processing γ -phosphoester- (1) and γ -phosphoramidate- (2 and 3) modified ATP analogues (Scheme 1).



Scheme 1. γ -Phosphate-modified ATP analogues: ATP-O-N₃ (1), ATP-NH-N₃ (2), and ATP-NH-biotin (3).

UBA1 is the initial enzyme in the ubiquitin-conjugation system, which modifies numerous target proteins by covalent attachment of ubiquitin (Ub).^[8] By cleaving the α - β -phosphoanhydride bond of ATP, UBA1 activates Ub through initial formation of Ub-adenylate, followed by the covalent attachment of Ub to UBA1 via a thioester bond.^[8,17] As ubiquitylation is involved in the regulation of numerous fundamental cellular processes,^[8,18] it is not surprising that deregulation of components of the ubiquitin-conjugation system contributes to the development of various human diseases including cancer and neurodegenerative disorders.^[19] Consequently, UBA1 has been considered a target in the treatment of various types of cancer.^[20] Therefore, a detailed understanding of the enzymatic activity of UBA1 and the downstream processes it is involved in is indispensable.

FAK, a protein tyrosine kinase (PTK), was chosen as an example of an enzyme that cleaves the β - γ -phosphoanhydride bond of ATP.^[21] It is an important intracellular signaling protein that regulates cell adhesion, shape, and motility by integrating signals from integrins and growth-factor receptors.^[22] Similarly to other PTKs, FAK transfers the γ -phosphate of ATP to the tyrosine residues of its substrates. FAK signaling can be triggered

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by cues from the extra-cellular matrix and is associated with the formation and turnover of cell adhesion structures, termed focal adhesions.^[21a,22] FAK protein expression is elevated in many highly malignant human cancers.^[23] It promotes changes in cell shape^[24] and formation of podosomes or invadopodia,^[25] which are involved in an invasive cell phenotype.^[26] Thus, understanding and monitoring FAK activity and signal transduction are highly desired.

In order to study the ability of γ -phosphoester- and γ -phosphoramidate-modified ATP analogues to support ATP-consuming reactions, we monitored the activity of UBA1 and FAK in vitro. Both enzymes showed a preference for phosphoramidate-modified ATP analogues over the phosphoester-modified counterpart, thus highlighting the significant effect of minor variations in ATP analogues for monitoring ATP utilization.

Results and Discussion

In order to investigate the influence of modifications at the γ -phosphate of ATP on enzymatic usage, we used a phosphoester-modified ATP analogue (1) and two phosphoramidate-modified ATP analogues (2 and 3; Scheme 1). The syntheses of 1 and 2 were as previously described.^[14] We synthesized 3 by treatment of disodium ATP with *N*-(6-aminohexyl)-*D*-biotinamide in the presence of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) in water^[13g] (for details, see the Supporting Information). The stabilities of all three ATP analogues under our assay conditions were confirmed by RP-HPLC (Figure S2 in the Supporting Information).

Turnover of the three γ -phosphate-modified ATP analogues by UBA1 was determined by performing an autoubiquitylation assay with E6AP (E6-associated protein).^[27] In this in vitro assay UBA1 activates Ub by forming a thioester bond between the C-terminal carboxyl group of Ub and the catalytic cysteine residue of UBA1 at the expense of ATP. Next, Ub is transferred to a cysteine residue of a ubiquitin-conjugating enzyme E2 (i.e., UbcH5b) and finally, it is covalently attached to a target protein by a ubiquitin-protein ligase E3 (Figure 1A).^[8,17] E6AP acts as both the E3 and the target protein, thereby resulting in modification of E6AP with several Ub moieties. This autoubiquitylation was monitored by SDS-PAGE and Coomassie staining (Figures 1B and S1). Thus, UBA1 activity (ATP consumption) leads to decreased levels of non-modified E6AP and free Ub and the formation of ubiquitylated E6AP (E6AP-Ub_n) with high molecular mass.

We observed that, similarly to the reaction with non-modified ATP, addition of phosphoramidate-modified ATP analogues resulted in the formation of ubiquitylated E6AP accompanied by equivalent decreases in the levels of free ubiquitin and non-modified E6AP. The level of highly ubiquitylated E6AP in the presence of 2 was comparable to that for ATP (Figure 1B, C and Figure S1). With 3, E6AP was somewhat less efficiently ubiquitylated, and no E6AP autoubiquitylation was observed with 1. The data clearly indicate that 2 and 3 are utilized by UBA1 for Ub activation, that is, UBA1 processes γ -phosphate-modified ATP analogues that bear a phosphoramidate-linkage (2 and 3) but not the analogue with a phosphoester linkage

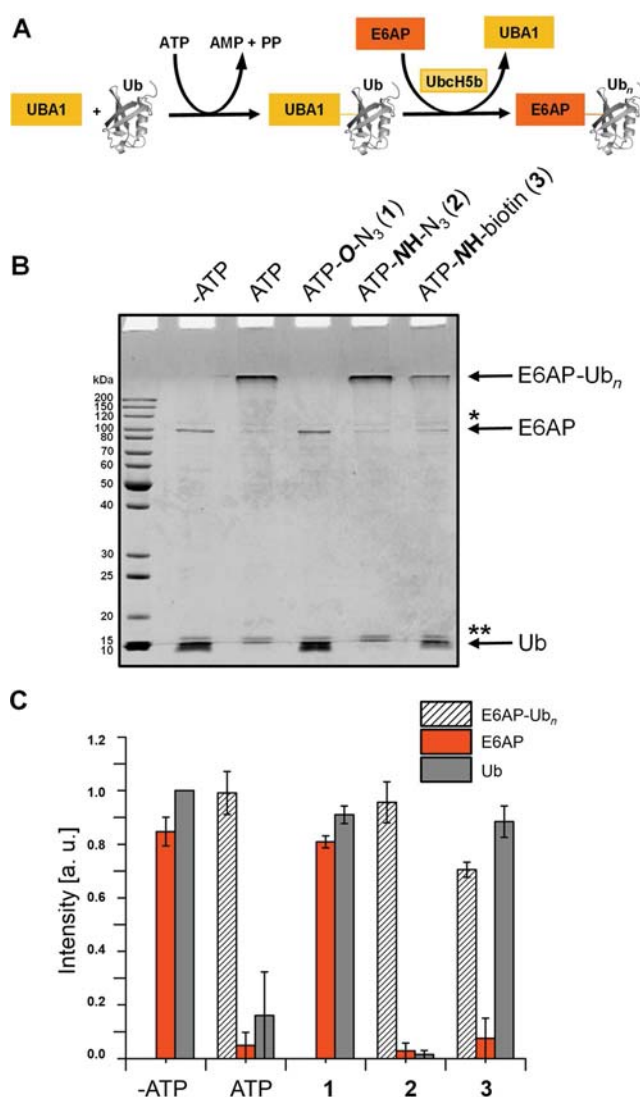


Figure 1. γ -Phosphate-modified ATP analogues in an E6AP autoubiquitylation assay. A) Scheme of UBA1 in vitro activity assay. Ubiquitin is activated by UBA1 in an ATP-dependent manner and then transferred to E6AP with the help of UbcH5b, thus resulting in poly-ubiquitylation (Ub_n) of E6AP. B) UBA1 consumption of γ -phosphate-modified ATP analogues monitored by E6AP ubiquitylation. ATP or analogue (500 μ M) was incubated with UBA1 (75 nM), UbcH5b (800 nM), E6AP (1.25 μ M), and ubiquitin (8 μ M) for 30 min at 37 °C. The products were analyzed by SDS-PAGE with Coomassie staining. C) Quantification of ubiquitylated E6AP (E6AP-Ub_n), unmodified E6AP, and free ubiquitin (Ub) in the assay. Values were normalized to the amount of free ubiquitin without ATP (mean \pm SEM of two independent experiments). *: UBA1; **: UbcH5b.

(1). Interestingly, we recently demonstrated that δ -phosphoester-modified adenosine tetraphosphates are consumed by UBA1.^[16]

Next, we studied FAK activity with the ATP analogues by using an in vitro kinase assay (Figure 2). FAK cleaves the β - γ -phosphoanhydride bond of ATP and transfers the γ -phosphate to a substrate tyrosine residue, for example, by autophosphorylation of tyrosine 397. For our assay design, the human FAK kinase domain (KD; aa 411–689) was fused to the sequence PEYFK (FIVE-tag) to obtain the recombinant enzyme hFAK-KD. Four copies of an FAK fragment containing Y397 (aa 378–406)

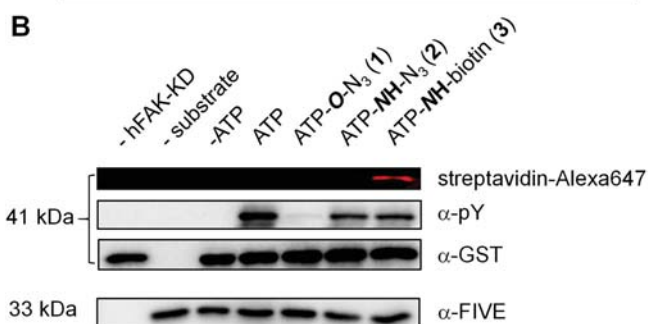
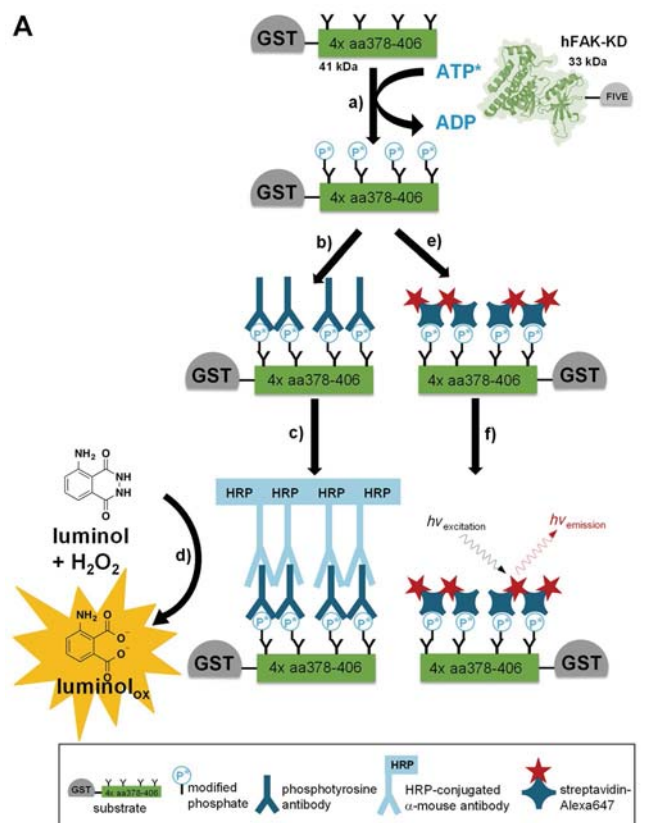


Figure 2. Utilization of γ -phosphate-modified ATP analogues by FAK. A) FAK in vitro kinase assay: a) GST-tagged substrate (160 nM; containing four tyrosines, Y) and the FIVE-tagged hFAK-KD (200 nM) were incubated with ATP or analogue (100 μ M) at 37 °C for 60 min; b) western blotting was followed by immunodetection with anti-phosphotyrosine antibody (α -pY), and c) Horseradish peroxidase (HRP)-conjugated secondary antibody; d) Luminol chemiluminescence was visualized upon oxidation by HRP. e) After western blotting, streptavidin-Alexa647 bound to biotin in **3**, and f) was detected by fluorescence readout. B) Utilization of ATP and analogues by FAK is visualized by western blotting and α -pY and fluorescence readout with streptavidin-Alexa647. Immunodetection with α -FIVE and α -GST verify the presence of equivalent amounts of the FIVE-tagged hFAK-KD and GST-FAK substrate in the respective samples.

were fused to a GST-tag to serve as the substrate. ATP (or analogue) was incubated with hFAK-KD and substrate at 37 °C for 60 min. The reaction products were analyzed by western blotting with antibodies against FIVE-tag, GST-tag, and phosphorylated tyrosine, as well as by streptavidin-Alexa647 fluorescence for the biotin on **3** (Figure 2). This detection method contrasts with that of our previously published assay,^[28] as it now allows

detection of the FIVE-tagged hFAK-KD and the GST-tagged FAK substrate in parallel. Phosphorylated substrate was detected for ATP, **2**, and **3** (see also Figure S3).

Time course experiments showed conversion of ATP, **2**, and **3** after just 5 min (Figure S3 C–E). Interestingly, the bulky biotin modification did not interfere with the reactivity of the anti-phosphotyrosine antibody. The phosphotyrosine signals were less pronounced for the phosphoramidate-modified ATP analogues (**2** and **3**) than for ATP, and no signal was observed for **1** (quantification in Figure S3 A).

Both UBA1 and FAK accepted the phosphoramidate-modified ATP analogues, but not the phosphoester-modified analogue. Acceptance of phosphoramidate-modified ATP analogues by a protein kinase is in accordance with the literature.^[13] Serine-threonine kinases have been shown to accept phosphoester-modified ATP analogues,^[13f,29] however, in these studies comparisons with phosphoramidate-modified counterparts were not performed. It is possible that (similarly to FAK here) these kinases might also show a strong preference for phosphoramidate-modified ATP analogues.

Phosphoester-modified dNTP and ATP have been reported to be utilized by DNA polymerases and UBA1, respectively, these were mainly tetra- and hexaphosphates (DNA polymerases)^[13d,30] and tetraphosphates (UBA1),^[16] not modified triphosphates as studied here. A possible explanation for the preference in utilizing phosphoramidate-modified ATP analogues is that the NH-moiety has a hydrogen-donating functionality that is absent in the ester functionalization.

Conclusion

We chose three γ -phosphate-modified ATP analogues to study their ability to be utilized by two ATP-cleaving enzymes of mechanistically distinct classes. Although UBA1 cleaves the α - β -phosphoanhydride bond of ATP whereas FAK transfers the γ -phosphate, they both favor the same γ -phosphate-modified ATP analogues, that is, they have a clear preference for phosphoramidate- over phosphoester-modified ATP. While no consumption of the phosphoester-modified ATP analogue **1** by the two enzymes was observed, the bulky phosphoramidate-modified ATP analogue **3** was readily accepted by both UBA1 and FAK. Furthermore, our data underline the high substrate specificity of the enzymes investigated in this study. Replacement of the NH moiety by oxygen on the γ -phosphate of ATP (compare **2** and **1**) abolished utilization. Therefore, small variations in the design of modified ATP analogues for monitoring ATP consumption can have a strong influence on enzymatic acceptance.

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- [1] F. Lipmann in *Advances in Enzymology and Related Areas of Molecular Biology, Vol. 1* (Eds. F. F. Nord, C. H. Werkman) Wiley, **1941**, pp. 99–162.
- [2] D. L. Stokes, W. R. Taylor, N. M. Green, *FEBS Lett.* **1994**, *346*, 32–38.
- [3] N. Hirokawa, Y. Noda, Y. Tanaka, S. Niwa, *Nat. Rev. Mol. Cell Biol.* **2009**, *10*, 682–696.
- [4] A. J. Roberts, T. Kon, P. J. Knight, K. Sutoh, S. A. Burgess, *Nat. Rev. Mol. Cell Biol.* **2013**, *14*, 713–726.
- [5] M. A. Hartman, J. A. Spudich, *J. Cell Sci.* **2012**, *125*, 1627–1632.
- [6] Y. Hisanaga, H. Ago, N. Nakagawa, K. Hamada, K. Ida, M. Yamamoto, T. Hori, Y. Arii, M. Sugahara, S. Kuramitsu, S. Yokoyama, M. Miyano, *J. Biol. Chem.* **2004**, *279*, 31717–31726.
- [7] S. Cusack, *Curr. Opin. Struct. Biol.* **1997**, *7*, 881–889.
- [8] A. Hershko, A. Ciechanover, *Annu. Rev. Biochem.* **1998**, *67*, 425–479.
- [9] D. Bunick, R. Zandomeni, S. Ackerman, R. Weinmann, *Cell* **1982**, *29*, 877–886.
- [10] G. Manning, D. B. Whyte, R. Martinez, T. Hunter, R. S. Sudarsanam, *Science* **2002**, *298*, 1912–1934.
- [11] a) C. J. Hastie, H. J. McLaughlan, P. Cohen, *Nat. Protoc.* **2006**, *1*, 968–971; b) A. Kornberg, J. F. Scott, L. L. Bertsch, *J. Biol. Chem.* **1978**, *253*, 3298–3304; c) H. Wegele, P. Muschler, M. Bunck, J. Reinstein, J. Buchner, *J. Biol. Chem.* **2003**, *278*, 39303–39310.
- [12] a) T. P. Geladopoulos, T. G. Sotiroidis, A. E. Evangelopoulos, *Anal. Biochem.* **1991**, *192*, 112–116; b) M. Catarinella, T. Grüner, T. Strittmatter, A. Marx, T. U. Mayer, *Angew. Chem. Int. Ed.* **2009**, *48*, 9072–9067; *Angew. Chem.* **2009**, *121*, 9236–9240.
- [13] a) K. D. Green, M. K. H. Pflum, *J. Am. Chem. Soc.* **2007**, *129*, 10–11; b) K. D. Green, M. K. H. Pflum, *ChemBioChem* **2009**, *10*, 234–237; c) S. Suwal, M. K. H. Pflum, *Angew. Chem. Int. Ed.* **2010**, *49*, 1627–1630; *Angew. Chem.* **2010**, *122*, 1671–1674; d) S. Kumar, A. Sood, J. Wegener, P. J. Finn, S. Nampalli, J. R. Nelson, A. Sekher, P. Mitsis, J. Macklin, C. W. Fuller, *Nucleosides Nucleotides Nucleic Acids* **2005**, *24*, 401–408; e) R. K. Suto, M. A. Whalen, B. R. Bender, R. G. Finke, *Nucleosides Nucleotides Nucleic Acids* **1998**, *17*, 1453–1471; f) S. E. Lee, L. M. Elphick, A. A. Anderson, L. Bonnac, E. S. Child, D. J. Mann, V. Gouverneur, *Bioorg. Med. Chem. Lett.* **2009**, *19*, 3804–3807; g) S. Serdjukow, F. Kink, B. Steigenberger, M. Tomás-Gamasa, T. Carell, *Chem. Commun.* **2014**, *50*, 1861–1863; h) A. E. Fouda, M. K. H. Pflum, *Angew. Chem. Int. Ed.* **2015**, *54*, 9618–9621; *Angew. Chem.* **2015**, *127*, 9754–9757; i) C. Senevirathne, D. M. Embogama, T. A. Anthony, A. E. Fouda, M. K. H. Pflum, *Bioorg. Med. Chem.* **2016**, *24*, 12–19.
- [14] S. M. Hacker, M. Mex, A. Marx, *J. Org. Chem.* **2012**, *77*, 10450–10454.
- [15] a) K. C. Facemyer, C. R. Cremona, *Bioconjugate Chem.* **1992**, *3*, 408–413; b) S. W. Kwon, S. C. Kim, J. Jaunbergs, J. R. Falck, Y. Zhao, *Mol. Cell. Proteomics* **2003**, *2*, 242–247.
- [16] S. M. Hacker, D. Pagliarini, T. Tischer, N. Hardt, D. Schneider, M. Mex, T. U. Mayer, M. Scheffner, A. Marx, *Angew. Chem. Int. Ed.* **2013**, *52*, 11916–11919; *Angew. Chem.* **2013**, *125*, 12133–12137.
- [17] a) I. Lee, H. Schindelin, *Cell* **2008**, *134*, 268–278; b) M. Scheffner, U. Nuber, J. M. Huijbrechtse, *Nature* **1995**, *373*, 81–83; c) A. Ciechanover, Y. Hod, A. Hershko, *Biochem. Biophys. Res. Commun.* **1978**, *81*, 1100–1105.
- [18] D. Komander, M. Rape, *Annu. Rev. Biochem.* **2012**, *81*, 203–229.
- [19] a) R. J. Deshaies, C. A. P. Joazeiro, *Annu. Rev. Biochem.* **2009**, *78*, 399–434; b) T. Ravid, M. Hochstrasser, *Nat. Rev. Mol. Cell Biol.* **2008**, *9*, 679–689; c) D. Rotin, S. Kumar, *Nat. Rev. Mol. Cell Biol.* **2009**, *10*, 398–409; d) R. L. Welchman, C. Gordon, R. J. Mayer, *Nat. Rev. Mol. Cell Biol.* **2005**, *6*, 599–609; e) D. Popovic, D. Vucic, I. Dikic, *Nat. Med.* **2014**, *20*, 1242–1253.
- [20] a) Y. Yang, J. Kitagaki, R.-M. Dai, Y. C. Tsai, K. L. Lorick, R. L. Ludwig, S. A. Pierre, J. P. Jensen, I. V. Davydov, P. Oberoi, C.-C. H. Li, J. H. Kenten, J. A. Beutler, K. H. Vousden, A. M. Weissman, *Cancer Res.* **2007**, *67*, 9472–9481; b) G. W. Xu, M. Ali, T. E. Wood, D. Wong, N. Maclean, X. Wang, M. Gronda, M. Skrtic, X. Li, R. Hurren, X. Mao, M. Venkatesan, R. Zavareh, T. Ketela, J. C. Reed, D. Rose, J. Moffat, R. A. Batey, S. Dhe-Paganon, A. D. Schimmer, *Blood* **2010**, *115*, 2251–2259.
- [21] a) D. D. Schlaepfer, C. R. Hauck, D. J. Sieg, *Prog. Biophys. Mol. Biol.* **1999**, *71*, 435–478; b) S. K. Mitra, D. D. Schlaepfer, *Curr. Opin. Cell Biol.* **2006**, *18*, 516–523.
- [22] a) S. K. Mitra, D. A. Hanson, D. D. Schlaepfer, *Nat. Rev. Mol. Cell Biol.* **2005**, *6*, 56–68; b) J. T. Parsons, *J. Cell Sci.* **2003**, *116*, 1409–1416.
- [23] W. G. Cance, J. E. Harris, M. V. Iacocca, E. Roche, X. Yang, J. Chang, S. Simkins, L. Xu, *Clin. Cancer Res.* **2000**, *6*, 2417–2423.
- [24] a) D. Ilic, B. Kovacic, S. MacDonagh, F. Jin, C. Baumbusch, D. G. Gardner, C. H. Damsky, *Circ. Res.* **2003**, *92*, 300–307; b) H. Haskell, M. Natarajan, T. P. Hecker, Q. Ding, J. Stewart, Jr., J. R. Grammer, C. L. Gladson, *Clin. Cancer Res.* **2003**, *9*, 2157–2165.
- [25] C. R. Hauck, D. A. Hsia, D. Ilic, D. D. Schlaepfer, *J. Biol. Chem.* **2002**, *277*, 12487–12490.
- [26] a) C. R. Hauck, D. A. Hsia, X. S. Puente, D. A. Cheresch, D. D. Schlaepfer, *EMBO J.* **2002**, *21*, 6289–6302; b) D. A. Hsia, S. K. Mitra, C. R. Hauck, D. N. Streblov, J. A. Nelson, D. Ilic, S. Huang, E. Li, G. R. Nemerow, J. Leng, K. S. R. Spencer, D. A. Cheresch, D. D. Schlaepfer, *J. Cell Biol.* **2003**, *160*, 753–767.
- [27] U. Nuber, S. E. Schwarz, M. Scheffner, *Eur. J. Biochem.* **1998**, *254*, 643–649.
- [28] S. M. Hacker, N. Hardt, A. Buntru, D. Pagliarini, M. Möckel, T. U. Mayer, M. Scheffner, C. R. Hauck, A. Marx, *Chem. Sci.* **2013**, *4*, 1588–1596.
- [29] R. Freeman, T. Finder, R. Gill, I. Willner, *Nano Lett.* **2010**, *10*, 2192–2196.
- [30] a) A. Sood, S. Kumar, S. Nampalli, J. R. Nelson, J. Macklin, C. W. Fuller, *J. Am. Chem. Soc.* **2005**, *127*, 2394–2395; b) P. B. Stranges, M. Palla, S. Kalachikov, J. Nivala, M. Dorwart, A. Trans, S. Kumar, M. Porel, M. Chien, C. Tao, I. Morozova, Z. Li, S. Shi, A. Aberra, C. Arnold, A. Yang, A. Aguirre, E. T. Harada, D. Korenblum, J. Pollard, et al., *Proc. Natl. Acad. Sci. USA* **2016**, *113*, E6749–E6756.