Actin activates *Pseudomonas aeruginosa* ExoY nucleotidyl cyclase toxin and ExoY-like effector domains from MARTX toxins

Alexander Belyy\(^1,2,\ast\), Dorothée Raoux-Barbot\(^1,\ast\), Cosmin Saveanu\(^3,\ast\), Abdelkader Namane\(^3\), Vasily Ogryzko\(^4\), Lina Worpenberg\(^1\), Violaine David\(^5\), Veronique Henriot\(^5\), Souad Fellous\(^5\), Christien Merrifield\(^5\), Elodie Assayag\(^1\), Daniel Ladant\(^1\), Louis Renault\(^5\) & Undine Mechold\(^1\)

The nucleotidyl cyclase toxin ExoY is one of the virulence factors injected by the *Pseudomonas aeruginosa* type III secretion system into host cells. Inside cells, it is activated by an unknown eukaryotic cofactor to synthesize various cyclic nucleotide monophosphates. ExoY-like adenylate cyclases are also found in Multifunctional-Autoprocessing Repeats-in-ToXin (MARTX) toxins produced by various Gram-negative pathogens. Here we demonstrate that filamentous actin (F-actin) is the hitherto unknown cofactor of ExoY. Association with F-actin stimulates ExoY activity more than 10,000 fold *in vitro* and results in stabilization of actin filaments. ExoY is recruited to actin filaments in transfected cells and alters F-actin turnover. Actin also activates an ExoY-like adenylate cyclase MARTX effector domain from *Vibrio nigripulchritudo*. Finally, using a yeast genetic screen, we identify actin mutants that no longer activate ExoY. Our results thus reveal a new sub-group within the class II adenyl cyclase family, namely actin-activated nucleotidyl cyclase (AA-NC) toxins.
**Pseudomonas aeruginosa** is an opportunistic human pathogen that causes severe infections in immune-compromised individuals and is a major cause of chronic infections in cystic fibrosis patients. Equipped with a type III secretion system (T3SS), *P. aeruginosa* can inject effector proteins directly into host cells where they contribute to virulence of the pathogen (for reviews see refs 1,2). Four different T3SS-delivered effectors have been characterized (exoenzyme T, Y, U and S), but new effectors were recently identified3. Exoenzyme Y (ExoY) is present in 89% of clinical isolates5. It was originally identified as an adenylate cyclase in 1998 due to amino-acid sequence homology with two well-characterized class II adenylate cyclase toxins, CyaA from Bordetella pertussis and edema factor from Bacillus anthracis6. Recent studies employing cultured cells revealed that substrate specificity of these enzymes is not restricted to ATP: edema factor and CyaA were shown to use uridine-5'-triphosphate (UTP) and cytidine-5'-triphosphate (CTP) as substrate6 while ExoY was shown to promote the intracellular accumulation of several cyclic nucleotides7,8 with a preference for cyclic GMP (cGMP) and cyclic UMP (cUMP) over cyclic AMP (cAMP) and cyclic CMP (cCMP) formation7.

ExoY was shown to induce cell death in a cellular infection model9 and severe, long-term lung damage in an animal infection model in rats10. At the molecular level, ExoYwas associated with actin is the common eukaryotic activator for a sub-group of the class II adenylyl cyclase toxin family19. Actin interacts with ExoY-TAP in *Saccharomyces cerevisiae*. To identify putative ExoY-binding proteins in yeast, plasmid-encoded ExoY with either a C-terminal TAP- or HA-tag (ExoY-TAP or ExoY-HA, respectively) was expressed in *S. cerevisiae*. To avoid toxic effects due to cyclic nucleotide accumulation, we used a catalytically inactive variant of ExoY, ExoYK81M (in which the Lys81 was mutated to Met5). Proteins co-purifying with the affinity purified bait protein were isolated by affinity purification (PAGE) (Supplementary Fig. 1), or processed by tryptic digestion and liquid chromatography–mass spectrometry (LC–MS/MS) extracts in vitro. Extracts from HeLa cells were used as positive control. We observed a dose-dependent stimulation of ExoY activity by yeast cell extracts, to levels that were similar to those measured when using HeLa cell extracts (Fig. 1a). Thus, we decided to use *S. cerevisiae* as a convenient experimental system to identify the putative yeast activator that was likely to be evolutionarily conserved in human cells.

**Figure 1 | Presence of an activator of ExoY in *Saccharomyces cerevisiae*.** (a) Activation of HF-ExoY by extracts from HeLa cells or *S. cerevisiae*. Reactions (50 µl) containing 1 µg ExoY were started by the addition of 2 mM ATP substrate and stopped after 30 min incubation at 30 °C and the amount of synthesized cAMP was measured. (b) Specific association of yeast Act1 to ExoYK81M. Log2 transformed LFQ scores for the proteins identified in the fraction that copurified with ExoYK81M, TAP (y axis) were represented as a function of the scores obtained for the control purification (ExoYK81M-HA, x axis). Black circles are the result of two or more superimposed grey circles. For clarity, only the 100 proteins with highest LFQ scores in the TAP purification are shown. Forty-five of these factors, including ExoY, were not identified in the control purification and are represented on the y axis alone. The dashed line was computed by linear regression for the 55 proteins having LFQ values in both experiments and indicates the trend for common contaminants in the affinity purification.

**Results**

An activator of ExoY is present in *Saccharomyces cerevisiae*. Arnoldo et al.20 have reported that overexpression of ExoY impairs yeast growth, suggesting that a cofactor required for ExoY catalytic activity should be present in yeast. To test this hypothesis, we prepared extracts from *Saccharomyces cerevisiae* BY4741 cells and measured adenylate cyclase activity of recombinant ExoY carrying an N-terminal His-Flag tag (HF-ExoY) in the presence of increasing amounts of yeast cell background and cell lines.

Recent whole-genome sequencing projects have identified ExoY-nucleotidyl cyclase modules among a variety of toxic Multifunctional-Autoprocessing Repeats-in-ToXin (MARTX) effector domains in numerous bacterial species of the *Vibrio* genus16 that represent emerging human or animal pathogens. These ExoY-like domains can be essential for virulence16. Elucidating the enzymatic specificities and molecular mechanisms of pathogenicity of ExoY and ExoY-like toxins may, therefore, help finding new therapeutic strategies against the toxicity and virulence of several bacterial pathogens.

Despite the progress in understanding downstream effects of ExoY activity, fundamental information on ExoY is lacking: similar to other bacterial soluble related cyclases such as CyaA and edema factor, ExoY is inactive in bacteria and is activated by an unknown eukaryotic cofactor after its delivery to the target cells5. Whereas the other class II adenylate cyclase toxins such as CyaA and edema factor are strongly activated upon interaction with calmodulin17,18, calmodulin is unable to stimulate ExoY enzymatic activity and the precise nature of the eukaryotic activator has remained elusive up to now. Here we report the identification of actin as the cofactor that activates *P. aeruginosa* ExoY and the ExoY-like module present in MARTX toxin of *Vibrio nigripulchritudo* in host cells. Our findings suggest that actin is the common eukaryotic activator for a sub-group of the class II adenylly cyclase toxin family19.

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**Figure 1** Presence of an activator of ExoY in *Saccharomyces cerevisiae*. (a) Activation of HF-ExoY by extracts from HeLa cells or *S. cerevisiae*. Reactions (50 µl) containing 1 µg ExoY were started by the addition of 2 mM ATP substrate and stopped after 30 min incubation at 30 °C and the amount of synthesized cAMP was measured. (b) Specific association of yeast Act1 to ExoYK81M. Log2 transformed LFQ scores for the proteins identified in the fraction that copurified with ExoYK81M, TAP (y axis) were represented as a function of the scores obtained for the control purification (ExoYK81M-HA, x axis). Black circles are the result of two or more superimposed grey circles. For clarity, only the 100 proteins with highest LFQ scores in the TAP purification are shown. Forty-five of these factors, including ExoY, were not identified in the control purification and are represented on the y axis alone. The dashed line was computed by linear regression for the 55 proteins having LFQ values in both experiments and indicates the trend for common contaminants in the affinity purification.
peptide/protein analysis. The raw data were then analysed by MaxQuant for protein identification and quantitative estimation of the specific enrichment of proteins in the experimental sample (ExoY<sup>K81M</sup>-TAP) as compared with the control (ExoY<sup>K81M</sup>-HA). While many abundant proteins were present in both samples to a similar degree, as estimated from the label-free quantitation score (LFQ<sup>a</sup>), ExoY was identified exclusively in the purification performed with ExoY<sup>K81M</sup>-TAP extracts, as expected. Another protein that was about 1,000 times more abundant in the ExoY<sup>K81M</sup>-TAP purification than in the control was yeast actin (Uniprot P60010, YFL039C, Act1), which showed an LFQ score close to the score of the tagged ExoY (Fig. 1b). Other factors were identified specifically in the ExoY<sup>K81M</sup>-TAP purification, but with much lower LFQ scores (see Supplementary Data 1). These results suggested a specific interaction of ExoY<sup>K81M</sup> with actin. Since actin is both specific to eukaryotic cells and one of the most highly conserved and abundant proteins in these cells, actin appeared to be an appropriate candidate for activating ExoY in mammalian cells.

**ExoY interacts with mammalian actin filaments in vitro.** To verify the interaction between ExoY and mammalian actin in vitro, we performed Ni-NTA agarose pulldowns using ExoY with a C-terminal Flag-His tag (ExoY-FH) and α-actin from rabbit skeletal muscle. Binding of ExoY to polymerized actin (F-actin, Fig. 2a) or to monomeric actin (G-actin) that was prevented from polymerization by the drug latrunculin A (Fig. 2b) was tested. While no binding of G-actin to ExoY could be observed (EA, Fig. 2b), quantitative densitometry analysis showed that 92% of F-actin and 100% of ExoY could be recovered by elution (elu) from samples that contained both proteins (EA, Fig. 2a). The unspecific binding of F-actin to Ni-agarose beads was low under the conditions tested as 85% of F-actin was found in the unbound fraction (fth) in samples containing only F-actin (A, Fig. 2a).

To confirm ExoY binding to F-actin, we performed high-speed cosedimentation assays using mixtures of ExoY and F-actin polymerized to steady state. High-speed centrifugation separated F-actin present in the pellet from G-actin present in the supernatant. Figure 2c shows that ExoY, which alone partitioned into the supernatant, was mostly found in the pellet fraction in the presence of F-actin, providing additional evidence that ExoY can interact with F-actin.

**Actin stimulates ExoY nucleotidyl cyclase activity in vitro.** We next tested whether purified actin could activate ExoY in vitro. We used buffer conditions allowing actin self-assembly to proceed above the critical concentration for polymerization. Highly pure non-muscle (cytoplasmic) actin isolated from human platelets (Cytoskeleton, Inc., designated here A-99) strongly stimulated the adenylate cyclase activity of ExoY (HF-ExoY), with a maximal activity reaching 120 μmol of cAMP min<sup>-1</sup> mg<sup>-1</sup> (Fig. 3a). Since expression of ExoY in transfected mammalian cells led to accumulation of cGMP to levels exceeding that of cAMP<sup>b</sup>, we also tested GTP as substrate. We found that the guanlylate cyclase activity of HF-ExoY was ~8 times higher than the adenylate cyclase activity in the presence of actin in vitro (Fig. 3a). The background activity without actin was estimated to be about 1 nmol min<sup>-1</sup> mg<sup>-1</sup> for cGMP and cAMP synthesis. Thus, the ExoY nucleotidyl cyclase activity was stimulated more than 10,000 fold by submicromolar concentrations of actin. Different mammalian actin isoforms (A-99 consisting of 85% β- and 15% γ-actin, or α-actin from rabbit skeletal muscle) were tested and all found to strongly activate ExoY-catalysed synthesis of cGMP in a concentration dependent manner (Supplementary Fig. 2), indicating that they were all effective activators of ExoY. Subsequent experiments were performed using α-actin from rabbit skeletal muscle purified in our laboratory and fully functional in actin polymerization assays (>95% pure, designated MA-L).

ExoY. To examine a possible dependence of ExoY activation on the different states of actin (ATP- versus ADP-bound, monomeric versus polymeric forms), we measured cGMP synthesis activity of ExoY at different actin concentrations below or above the critical concentrations that favor actin self-assembly in these different nucleotide states. Measurements were performed at increasing...
concentrations of actin that was initially loaded with either Mg-ATP or Mg-ADP. A similar maximal activity of 1,000–1,200 μmol of cGMP min⁻¹ mg⁻¹ was obtained with both ATP- and ADP-bonded actin (Fig. 3b). In contrast, the actin concentrations required for half maximal activation of ExoY (K_{1/2}) were dependent on the bound nucleotides: half maximal ExoY activation was obtained using 0.2 μM of ATP-loaded actin (Fig. 3b), just above the critical concentration of 0.1 μM at which Mg-ATP-actin spontaneously polymerizes with salt. Conversely, half-maximal ExoY activation was obtained at about 2.4 μM ADP-loaded actin (Fig. 3b), a value close to the critical spontaneous polymerizing concentration (about 2 μM) of Mg-ADP-actin. Altogether, these results suggest that the maximal activation of ExoY by actin was correlated with F-actin formation.

**Effect of latrunculin A and G-actin-binding proteins.** We examined whether proteins or molecules known to bind to G-actin and to inhibit elongation and/or spontaneous nucleation, alter the activation of ExoY by actin. We used latrunculin A or the abundant G-actin-binding proteins profilin and thymosin-B4 (Tβ4). These three molecules are known to bind to distinct G-actin interfaces: (i) The small macrolide latrunculin A inhibits actin self-assembly by binding (K_{d} ~ 0.2 μM) to a cleft located on the pointed face of G-actin. (ii) Profilin binds (K_{d} ~ 0.1 μM) to the opposite face of monomers, called barbed face, and favors in vivo the unidirectional elongation of the most-dynamic barbed ends of filaments. In vitro, G-actin profilin complexes inhibit actin spontaneous nucleation and thus polymerization in the absence of actin nuclei or filament seeds. (iii) Tβ4 acts as a major G-actin-sequestering polypeptide in cells. Here we used a chimeric β-thymosin domain, chim2-Tβ4 (a chimera between bovine Tβ4 and Drosophila ciboulot made of β-thymosin repeats), which sequesters G-actin monomers with higher affinity than Tβ4 (K_{d} ~ 0.5 μM versus 2 μM). As with Tβ4, the binding interface of chim2-Tβ4 with actin monomers is extensive, covering both the barbed and pointed faces.
Actin monomers were saturated with these molecules to inhibit the spontaneous nucleation or polymerization of actin. As shown in Fig. 3c, latrunculin A, profilin and chim2-TP4, all efficiently inhibited ExoY activation by actin at concentrations that fully activate the enzyme in control assays. These data thus provide additional indications that filamentous actin is the preferred activator of ExoY.

**ExoY binding along actin filaments alters their turnover.** We examined whether ExoY interaction with actin alters the intrinsic or regulated dynamics of actin self-assembly *in vitro*. To avoid indirect effects of ATP depletion due to ExoY activity, we used the inactive variant ExoYK81M in assembly/disassembly kinetic studies with G-/F-actin-ATP. Alternatively, wild-type ExoY was used in kinetic studies with G-/F-actin-ADP, since ADP is not an ExoY substrate. The kinetics of polymerization or depolymerization were monitored by following the increase or decrease, respectively, of pyrene-actin fluorescence intensity (pyrenyl-labelled actin subunits exhibit higher fluorescence when incorporated in filaments than free in solution). In polymerization assays, ExoYK81M slightly accelerated the rate of G-actin-ATP-Mg or G-actin-ADP-Mg self-assembly (Fig. 4a, Supplementary Fig. 4), confirming that ExoY interacts with actin without preventing actin self-assembly. Yet, this stimulation of G-actin-ATP/ADP polymerization by ExoY was detected only at high ExoY concentrations (in μM range). Besides, ExoY-stimulated actin polymerization was abolished when G-actin was saturated by profilin (Fig. 4a). In eukaryotic cells, the polymerization competent G-actin pool is mainly bound to profilin^{22,27}. Therefore, these results indicate that ExoY is unlikely to stimulate actin polymerization in host cells. To delineate the interaction of ExoY with F-actin, we performed filament disassembly assays monitored from free barbed- or pointed-ends. As shown in Fig. 4b, ExoYK81M inhibited spontaneous F-actin disassembly induced by dilution. This inhibition was also observed when barbed ends were capped by gelsolin (Fig. 4b), thus excluding the possibility that ExoY inhibited disassembly by binding to barbed ends. These results, as well as the absence of ExoY effects on pointed and barbed-end elongation rates (Supplementary Fig. 5), indicate that ExoY binds along the sides of filaments. We considered two key regulatory proteins, which are ubiquitous among eukaryotic cells: the Arp2/3 complex and actin-depolymerizing factor (ADF). The Arp2/3 complex, upon its activation by VCA domains of the WASP family proteins, binds to the side of a pre-existing filament where it catalyses actin filament branching^{22,27,30}. ADF/cofilin proteins, present at micromolar concentrations in eukaryotic cells, bind cooperatively and preferentially to F-actin-ADF subunits along filaments (Kd~0.1 μM), severing aged filaments, and enhancing their disassembly and turnover^{22,27,31}.

In experiments with the Arp2/3 complex, we performed actin polymerization assays using G-actin saturated by profilin to approach a more physiological context. As shown in Fig. 4d, the acceleration of G-actin-ATP polymerization by VCA-activated Arp2/3 (25–35 nM) was inhibited by ExoYK81M at concentrations of 100 nM and higher. This demonstrates that ExoY antagonizes the binding of the activated Arp2/3 complex along filaments and hence VCA-Arp2/3 regulation.

In dilution-induced F-actin-ADP disassembly assays (Fig. 4e), ExoY or ExoYK81M (100 nM) were able to completely inhibit the acceleration of filament disassembly promoted by ADF (4 μM) at a low ExoY:ADF ratio of 1:40. The sub-stoichiometric ratio of ExoY with respect to ADF and the respective Kd for F-actin binding suggested that the complete inhibition of ADF activity by ExoY was not achieved by a direct competition for binding to F-actin. To investigate how ExoY inhibits the disassembly of F-actin mediated by ADF, we examined whether ExoY binding to F-actin imposes cooperative conformational perturbations on the F-actin structure and thus stabilizes filaments in a conformation incompatible with ADF binding and severing activity. We used the drug phallodin, which binds with high affinity to F-actin. Compared with native filaments, phallodin-stabilized filaments appear stiffer and exhibit an altered conformation^{32} that inhibits the cooperative binding and activity of ADF. To test whether ExoY imposes cooperative conformational perturbations on F-actin, we measured the binding affinity of ExoYK81M to phallodin-modified filaments using cosedimentation assays. For three different conformational states of F-actin: native, phallodin-bound (Supplementary Fig. 6a), or F-actin-ADP-BeF3 (Fig. 4c), the affinity of MBP-ExoYK81M for filaments remained unchanged (Kd~1.6–1.8 μM). Thus, ExoY binding does not appear to induce a particular cooperative conformational change in F-actin. Then we examined whether a pre-incubation of actin filaments with MBP-ExoYK81M altered subsequent binding of ADF to F-actin. We used the low MBP-ExoYK81M:ADF ratios that antagonized ADF activity in the depolymerization assays of Fig. 4e. We found no significant change of ADF binding to F-actin at a low MBP-ExoYK81M:ADF ratio of 1:40 and a slight decrease (about 16%) at a 1:6.7 ratio (Supplementary Fig. 6b). This suggests that ExoY binding to F-actin antagonizes ADF depolymerizing activity by preventing its cooperative binding along actin-ADF filaments.

**F-actin cell content increases upon ExoY recruitment to cables.** To our knowledge, the localization of ExoY in eukaryotic cells was not previously reported and is of particular interest in light of its direct interaction with F-actin *in vitro*. To avoid toxic effects of ExoY when expressed in eukaryotic cells, the catalytically inactive variant ExoYK81M fused to AcGFP (a monomeric green fluorescent protein) was expressed in transiently transfected HeLa cells (a human cell line of epithelial origin). Co-localization of ExoYK81M-AcGFP with F-actin was first analysed using spinning disk confocal microscopy of phallodin-stained cells. Phallodin was conjugated to a far-red-670 nm fluorophore to ensure signal
Figure 4 | ExoY association with F-actin can interfere with the regulation of filament dynamics by eukaryotic cytoskeletal proteins. (a) MBP-ExoY<sub>K81M</sub> mediated acceleration of G-actin-ATP-Mg self-assembly rate and its inhibition by profilin. Four micromolar G-actin-Mg-ATP (3% pyrenyl labelled) was polymerized in the absence (continuous lines) or presence (dashed lines) of 12 µM profilin at the indicated concentrations (µM) of MBP-ExoY<sub>K81M</sub>. MBP by itself (4 µM, brown) did not stimulate polymerization. (b) ExoY<sub>K81M</sub> binding to filaments inhibits their spontaneous disassembly kinetics from free barbed- or pointed-ends. Disassembly from free barbed-ends (continuous lines): F-actin (2 µM, 50% pyrenyl) at steady state was diluted to 50 nM in the presence of 0 to 250 nM ExoY<sub>K81M</sub>. Disassembly from pointed-ends (dotted lines): filaments with their barbed-ends capped by Gelsolin (10 µM actin, 33.3 nM Gelsolin) at steady state were diluted to 300 nM in the presence of 0 to 250 nM ExoY<sub>K81M</sub>. (c) Binding affinity of MBP-fused ExoY and ExoY<sub>K81M</sub> for F-actin (MA-L) measured in high-speed cosedimentation assays. Left panels: Representative Coomassie blue stained SDS–PAGE (10%) gel images from the supernatant (S) and pellet (P) fractions using 3 µM actin. The supernatant (S) and pellet (P) fractions using 3 µM actin. F-actin (MA-L) measured in high-speed cosedimentation assays. Left panels: Representative Coomassie blue stained SDS–PAGE (10%) gel images from the supernatant (S) and pellet (P) fractions using 3 µM actin. F-actin (MA-L) measured in high-speed cosedimentation assays. Left panels: Representative Coomassie blue stained SDS–PAGE (10%) gel images from the supernatant (S) and pellet (P) fractions using 3 µM actin. Right panel: The increasing amounts of MBP-ExoY<sub>K81M</sub> bound to filaments were measured by densitometry, normalized and fitted to derive the equilibrium dissociation constant (K<sub>d</sub>) of the proteins for F-actin-ADP-BeF<sub>3</sub>. Error bars are s.e. (n ≥ 3). (d) ExoY<sub>K81M</sub> inhibits the acceleration of filament formation induced by VCA-activated Arp2/3 complex. Three micromolar G-actin-Mg-ATP (3% pyrenyl) was polymerized with 7.5 µM profilin, 0.2 µM NWASP-VCA, in the absence (black) or presence of 35 nM Arp2/3 and 0 to 600 nM ExoY<sub>K81M</sub>. (e) ExoY<sub>K81M</sub> inhibits the acceleration of actin-F-actin disassembly promoted by ADF. F-actin-ADF (9 µM, 50% pyrenyl) at steady state was diluted to 4 µM and preincubated for 2 min with 0 to 630 nM ExoY<sub>K81M</sub> (continuous lines) or ExoY<sub>K81M</sub> (dotted lines) before depolymerization assays without ADF (0 nM ExoY<sub>K81M</sub>, black) or with 4 µM ADF (grey, cyan or blue curves).

separation from AcGFP. We observed the recruitment of ExoY to F-actin rich structures in particular along the plasma membrane, to ruffles and to actin cables (Fig. 5a). Calculated Pearson’s correlation coefficient (PCC) of 0.57 ± 0.15 (mean ± s.d.; n > 15 cells) showed significant co-localization of ExoY<sub>K81M</sub>-AcGFP with F-actin, whereas the transfection of AcGFP alone led to a negative PCC (Fig. 5b). To show ExoY co-localization with actin fibres, we next performed cotransfection with mCherry-Vinculin, a protein of focal adhesions. Contractile actin fibres are often connected to focal adhesion at their ends. Using total internal reflection fluorescence (TIRF) microscopy, we observed that ExoY<sub>K81M</sub>-AcGFP labelled stress fibres especially at vinculin labelled focal

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adhesion (Fig. 5d), whereas GFP alone was uniformly distributed (Supplementary Fig. 7). Quantifications of phalloidin staining of spinning disk confocal microscopy pictures indicated additionally that F-actin content was increased in ExoY-expressing cells. The mean fluorescent intensities of F-actin over all section surfaces (from bottom to top within each cell), or of actin stress fibres measured at the bottom slices, were higher than in control AcGFP-expressing cells (Fig. 5c). In agreement with our biochemical analysis of ExoY in F-actin disassembly assays, these data establish that the bacterial toxin can bind to and stabilize microfilaments in host cells in the presence of myriad actin-binding regulatory proteins.

**Activation of a *Vibrio* ExoY-like protein by actin.** We further examined whether actin could activate other putative ExoY-like cyclases that are present in a number of MARTX toxins from

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**Figure 5 | ExoY co-localizes with actin cables and increases F-actin cell content.** (a) Staining of F-actin (670 nm fluorescent phalloidin) in HeLa cells transfected with ExoYK81M-AcGFP construct. Scale bars, 10 μm. Spinning disk confocal microscopy pictures show co-localization of ExoYK81M-AcGFP with F-actin from the bottom slices or the top ones and increased phalloidin staining in comparison to the control (AcGFP). (b) The co-localization of ExoYK81M-AcGFP with F-actin was estimated by measuring the average Pearson’s correlation coefficient (± s.d.; n≥ 15 cells) between AcGFP and 670 nm fluorescence signals in individual whole cells (without extracellular regions). (c) Quantification of F-actin within whole cells (from bottom to top slices in each cell) and of actin stress fibres at the bottom slices in ExoYK81M-AcGFP- or AcGFP-expressing cells. Data are mean fluorescent intensity per pixel ± s.d. (n≥15 cells; *P < 0.001; two tail Student’s t-test). (d) TIRF pictures of HeLa cell expressing ExoYK81M-AcGFP and mCherry-vinculin. Scale bars, 5 μm. ExoYK81M-AcGFP-labelled stress fibres terminated at vinculin-labelled focal adhesions (arrows).
pathogenic proteobacterial species present in the genera *Burkholderia*, *Providencia*, or *Proteus* (Fig. 6a). For this, we selected the ExoY-like effector module from the MARTX toxin encoded by the virulence-associated plasmid pA	extsubscript{Sfa1} from *V. nigrilucitridito* 

Figure 7 | Actin mutant alleles affecting the activation of ExoY. (a) Drop tests of serial dilutions of *S. cerevisiae* strains expressing wild-type (wt) actin (SC489), D25Y/D222G (SC690) or D25N (SC691) and p1593 for galactose-induced expression of ExoY or the corresponding vector control YEpGal555. Cell suspensions were normalized to an OD600 of 1.0 and 5-fold serial dilutions were applied as 3 μl drops on SD or SG-agar plates. (b) Western blot analysis to verify expression of Myc-ExoY in SC690 and SC691 with anti-Myc or anti-RPS9 (loading control). (c) Western blot analysis to verify expression of actin in SC489 (1), SC690 (2) and SC691 (3) with anti-actin or anti-RPS9 (loading control). Uncropped images of (b,c) are shown in Supplementary Fig. 10.

Identification of actin mutants that fail to activate ExoY. To further delineate the molecular mechanism of activation of ExoY by actin, we attempted to identify actin mutants with impaired ability to activate ExoY. For this purpose, we used a recently developed yeast genetic screen, in which the wild-type actin of *S. cerevisiae* is replaced by actin variants expressed from a plasmid. *S. cerevisiae* contains a single actin gene (*act1*), which can be deleted provided the strain harbours a plasmid (with an URA3 marker) carrying a complementary copy of wild-type actin (*S. cerevisiae*::LEU2 + pACT1 [URA3]). This recombinant strain was then transformed with a pool of plasmids carrying the HIS3 auxotrophic marker and expressing actin variants generated by *in vitro* mutagenesis (see Methods). Subsequently, the URA3-containing plasmid was eliminated (by selection on 5-fluoroorotic acid containing medium) and the
resulting cells, therefore, expressed only the actin variants encoded by the HIS3-plasmid. These cells were transformed with a plasmid (p1654) expressing ExoY (Myc-ExoY-NanoLuc) under the control of the galactose-inducible GAL1 promoter and cells that tolerated ExoY expression were isolated. Twenty-one ExoY-resistant colonies (with unimpaired ExoY expression levels) were selected and found to harbour two different actin mutant alleles: 14 mutants had a double substitution, D25Y and D222G, and 7 had a single modification D25N. Figure 7a compares growth of S. cerevisiae strains expressing wild-type actin (SC489), mutant D25Y/D222G (SC690) or mutant D25N (SC691) and ExoY in drop tests of sequentially diluted cultures. Whereas ExoY expression (that is, in the presence of galactose) totally inhibited the growth of the wild-type strain, it did not affect the growth of the yeast cells harbouring either one of the two actin mutants. We verified ExoY expression in the actin mutant strains by western blots with anti-Myc antibodies (Fig. 7b). Both actin variants were similarly expressed (Fig. 7c) and supported growth of S. cerevisiae: the double mutant grew slightly more slowly in SD minimal medium as compared with the wild type and the single mutant (doubling time 3 h versus 2.5 h, respectively) and was slightly affected under different stress conditions tested: at 15 or 37°C or in the presence of 1M NaCl (Supplementary Fig. 9).

Crude extracts of the different S. cerevisiae strains were then prepared and tested for their ability to activate ExoY in vitro. While extracts from wild-type cells strongly stimulated ExoY, no activity of ExoY was detected in extracts from yeasts expressing the actin mutants (Table 1). In addition, we found that the actin mutations D25Y/D222G or D25N also largely impaired the activation of the Vibrion enzyme Vn-ExoY in vitro (Table 1).

**Discussion**

Bacterial toxins that use substrates not unique to eukaryotic organisms need to be kept inactive within the bacterial pathogen in order to prevent detrimental effects to the native host. Once they enter the eukaryotic host cell, the toxin is usually activated by a host cell cofactor that typically represents a specific and abundant protein/marker of their hosts. Actin fits these criteria perfectly well: actin is absent from bacteria, found in essentially all eukaryotic cells where it is one of the most abundant proteins, and despite evolutionary separation by billions of years, S. cerevisiae and human actin share 87% amino-acid sequence identity. These features make actin also a frequent target of bacterial invasion. They enter the eukaryotic host cell, the toxin is usually activated in order to prevent detrimental effects to the native host. Once organisms need to be kept inactive within the bacterial pathogen in vitro mechanisms of activation of ExoY and YopO by F- and G-actin are different (see Figs 1, 4 and 5).

**Table 1 | Activation of ExoY or VnExoY-L by S. cerevisiae extracts expressing wild-type or mutant actin.**

|                  | No extract | S. cerevisiae extracts |
|------------------|------------|------------------------|
|                  | SC489 Wild type | SC690 D25Y/D222G | SC691 D25N |
| ExoY-catalysed synthesis of cGMP (nmol min\(^{-1}\)mg\(^{-1}\)) | <5 | 2,950 ± 330 | <5 |
| VnExoY-L-catalysed synthesis of cAMP (nmol min\(^{-1}\)mg\(^{-1}\)) | <5 | 2,800 ± 210 | 19 ± 8 |

Reactions containing 0.5 μg of ExoY-FH or VnExoY-L-FH and 50 μg of S. cerevisiae cell extracts were started by the addition of 2 mM GTP or ATP substrate and incubated for 30 min at 30°C.

Using a yeast genetic screen (Fig. 7), we further identified an actin mutation (D25N) that blunts actin’s ability to activate ExoY. D25 in actin subdomain 1 is a charged residue solvent-exposed in F-actin and can thus be part of the contact interface of F-actin-binding proteins. These data not only confirm that actin is an activator of ExoY but demonstrate that actin is the only activator of ExoY present in yeast. We can thus exclude the possibility that a contaminant present in the actin preparations could be responsible for activation of ExoY in our in vitro assays.

Our results support the view that polymerization of G-actin into F-actin is critical for maximum activation of ExoY. First, the maximal activation of ExoY by actin-ATP or actin-ADP was correlated with F-actin formation in each nucleotide state (Fig. 3b). Second, ExoY activation by actin was strongly antagonized by different G-actin-binding proteins, such as profilin, or a Tβ4-derivative protein with similar sequestering activity as Tβ4 (chim2-Tβ4), or by latrunculin A that prevents actin polymerization (Fig. 3c). Finally, ExoY binds along naked filaments with a low micromolar affinity (Kd of about 1 ± 0.2 μM, Fig. 4c), which should allow efficient competition in vivo with many eukaryotic cytoskeletal side-binding proteins that also bind along filaments with low micromolar affinity.

The co-localization of ExoY-GFP with F-actin rich structures confirmed that ExoY indeed associates with actin filaments within cells (Fig. 5). ExoY thus represents to our knowledge the first example of a bacterial toxin that is activated by F-actin. G-actin has been shown previously to activate a bacterial toxin secreted by the T3SS namely YopO/YpkA, a multidomain protein from Yersinia species (Y. enterolocolitica and Y. pseudotuberculosis, respectively), which is involved in the disruption of the actin cytoskeleton. In contrast, P. aeruginosa ExoY binding to F-actin inhibits the spontaneous or regulated dynamics of F-actin disassembly in vitro (Fig. 4b,e) increasing the microfilament content in transfected cells (Fig. 5). YopO binding with an actin monomer induces autophosphorylation and activation of its N-terminal serine/threonine kinase domain. In the YopO-G–actin complex, the bound actin is sequestered from polymerization and used subsequently as bait to recruit, phosphorylate and thus misregulate various host actin-regulating proteins. The mechanisms of activation of ExoY by YopO by F- and G-actin, respectively, are, therefore, likely different.

While most of the ExoY-related toxicity in infected cells depends on its nucleotidyl cyclase activity, its direct binding to actin filaments could additionally alter normal host cell homeostasis. We showed that in vitro (Fig. 4e), ExoY (as low as about 120 and 900 μmol min\(^{-1}\)mg\(^{-1}\)) for cAMP and cGMP synthesis, respectively (Fig. 3a). The higher guanylate cyclase activity compared with the adenylate cyclase one is in agreement with the preferential accumulation of cGMP over cAMP observed in vivo. The corresponding kcat for cGMP synthesis is approaching 1,000 s\(^{-1}\) and, therefore, within the same order of magnitude as the catalytic rates measured for cAMP synthesis for the related cyclase toxins CyaA and edema factor, when activated by calmodulin, their common eukaryotic activator.

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50 nM) can antagonize the cooperative activity of ADF at sub-molar ratios of ExoY with respect to the regulatory cytoskeletal side-binding protein. Even though ExoY may be present at lower concentrations in host cells, its binding along actin filaments might nevertheless contribute to destabilizing precise spatial and temporal regulation of actin dynamics in eukaryotic cells. Huber et al.15 have recently observed that at 3 h post-infection, a P. aeruginosa TSSS effector strain manifesting only ExoY showed slightly increased human endothelial cell spreading, suggesting a stabilization of the actin cytoskeleton. It will thus be interesting to examine in more details, the actin cytoskeleton dynamics of host cells upon infection with bacteria expressing catalytically inactive ExoY or ExoY-like proteins alone or together with other toxins affecting actin cytoskeleton regulation (ExoO, ExoT from P. aeruginosa, actin cross-linking (ACD) or Rho-GTPase inactivation domain (RID) from various MARTX toxins of the Vibrio genus16). Given that ExoS and ExoT disrupt actin filaments, cytotoxicity of ExoY might be self-limited by the interplay between P. aeruginosa TSSS toxins. Such an interplay between ExoY and ExoO or ExoT acting via on actin cytoskeleton integrity may explain why a mutant strain of P. aeruginosa injecting only ExoY as TSSS effector was found more potent for inducing high CAMP levels in infected human endothelial cells than the wild-type or mutant strains expressing all three TSSS toxins or ExoY with either ExoS or ExoT, respectively.15

ExoY-like modules are frequently found among the effector domains of MARTX toxins in multiple bacterial species of the Vibrio genus16, which represent emerging human or animal pathogens. In addition, ExoY-like proteins can be found in various other Gram-negative pathogenic bacteria from the genus Providencia, Burkholderia or Proteus (Fig. 6a). Here we showed that VnExoY-L, a rather distantly related ExoY-like module from V. nigripulchritudo, was also strongly stimulated (more than 10,000 fold) by actin and efficiently synthesized cAMP but not cGMP (Fig. 6b). The lack of guanylate cyclase activity is in agreement with the results obtained with the V. vulnificus ExoY-like module16, a close homologue of VnExoY-L (> 75% sequence similarity, Fig. 6a and Supplementary Table 2), and may thus reflect a more general difference regarding the nucleotide substrate specificities between the P. aeruginosa ExoY and other ExoY-like proteins found in MARTX toxins similar to those present in the Vibrio genus (Fig. 6a and Supplementary Fig. 8).

Actin thus represents a common eukaryotic activator for several exoenzymes (Fig. 6a) within the class II adenylyl cyclase ExoY-like proteins found in MARTX toxins similar to those of Vibrio genus16, which represent emerging human or animal pathogens. In addition, ExoY-like proteins can be found in various other Gram-negative pathogenic bacteria from the genus Providencia, Burkholderia or Proteus (Fig. 6a). Here we showed that VnExoY-L, a rather distantly related ExoY-like module from V. nigripulchritudo, was also strongly stimulated (more than 10,000 fold) by actin and efficiently synthesized cAMP but not cGMP (Fig. 6b). The lack of guanylate cyclase activity is in agreement with the results obtained with the V. vulnificus ExoY-like module16, a close homologue of VnExoY-L (> 75% sequence similarity, Fig. 6a and Supplementary Table 2), and may thus reflect a more general difference regarding the nucleotide substrate specificities between the P. aeruginosa ExoY and other ExoY-like proteins found in MARTX toxins similar to those present in the Vibrio genus (Fig. 6a and Supplementary Fig. 8).

Actin thus represents a common eukaryotic activator for several exoenzymes (Fig. 6a) within the class II adenylyl cyclase ExoY-like proteins found in MARTX toxins similar to those present in the Vibrio genus (Fig. 6a and Supplementary Fig. 8).

Methods

Strains and growth conditions. Strains, plasmids and primers are described in Supplementary table 1. E. coli strains were grown in lysogeny broth. Ampicillin (100 μg ml−1) was added for plasmid maintenance in E. coli. S. cerevisiae strains were grown in yeast extract peptone dextrose or yeast extract peptone galactose media or in minimal media containing yeast nitrogen base without amino acids (Difco) containing galactose (Sc) or glucose (Nd) supplemented with uracil, histidine, tryptophane and/or adenine if required. Glucose or galactose was present at 2%. Hygromycin (Sigma) was present at 200 μg ml−1, to maintain plasmids in yeast. S. cerevisiae strains were transformed using the lithium-acetate method17.

The plasmid for expression of HF-ExoY under control of the arabinose-inducible promoter P_{ara}/(pUM447) was cloned as follows: Primers UM248 and UM254 were used to PCR-amplify exoY from P. aeruginosa PA01 chromosomal DNA. The EcoRI, XbaI digested fragment was used to replace the EcoRI/XbaI fragment from pUM447 by that of pUM460 that was digested with BglII and XhoI. pUM518 for the expression of ExoYK81M fused to the N-terminus of AcGFP in mammalian cells was created by insertion of a NheI/XhoI digested PCR fragment that was amplified from pUM350 and pUM462 into pAGFP-N1. pUM522 for the expression of Vn-ExoY-L-FH under the temperature sensitive cI repressor (cI857) was constructed by replacing the Ncol/KpnI fragment expressing ExoY from pUM460 by that of pUM497. The fusion constructs of ExoY/ExoYK81M with an N-terminal Myc-tag in yeast was created by insertion of a XhoI/SfiI digested PCR fragment that was amplified from pUM445 or pUM502, respectively. The BglII, XhoI digested fragments were inserted into a modified pEX-6-P1 (ref. 47) digested with BamHI and XhoI.

p1593 for the expression of ExoY with a N-terminal Myc-tag in yeast was created by insertion of a XhoI/KpnI digested PCR fragment that was amplified from pUM445 using primers 1259 and 1260 into YEplac53 (ref. 35). ExoY expressed from p1593 was C-terminally fused to NanoLuciferase to allow quantification of expression. The fragment coding for NanoLuc was PCR-amplified using primer UM254 and UM255 and cloned as NcoI/XbaI fragments into the same sites of plasmid pTRCAG. The plasmid for expression of HF-ExoY under control of the arabinose-inducible promoter P_{ara}/(pUM447) was cloned as follows: Primers UM248 and UM254 were used to PCR-amplify exoY from P. aeruginosa PA01 chromosomal DNA. The EcoRI, XbaI digested fragment was used to replace the EcoRI/XbaI fragment from pUM447 by that of pUM460 that was digested with BglII and XhoI. pUM518 for the expression of ExoYK81M fused to the N-terminus of AcGFP in mammalian cells was created by insertion of a NheI/XhoI digested PCR fragment that was amplified from pUM350 and pUM462 into pAGFP-N1. pUM522 for the expression of Vn-ExoY-L-FH under the temperature sensitive cI repressor (cI857) was constructed by replacing the Ncol/KpnI fragment expressing ExoY from pUM460 by that of pUM497. The fusion constructs of ExoY/ExoYK81M with an N-terminal Myc-tag in yeast was created by insertion of a XhoI/KpnI digested PCR fragment that was amplified from pUM445 or pUM502, respectively. The BglII, XhoI digested fragments were inserted into a modified pEX-6-P1 (ref. 47) digested with BamHI and XhoI.

Plasmid p1387 was used for random mutagenesis of S. cerevisiae act1 and was created from p1182 by removing the Caal site located within act1 to construct a plasmid with unique ClaI and Sall sites flanking the gene’s second exon coding for amino acid 4 to the C’end of Act1.

Protein purification. ExoY-FH and VnExoY-L-FH were purified by nickel affinity chromatography under denaturing conditions (in the presence of 8 M urea) from the non-soluble protein fraction obtained from 1 liter cultures of E. coli BLR (pUM440) or (pUM522), respectively. Proteins were expressed from the L_P1 promoter controlled by the temperature sensitive cI repressor (cI857), which was induced by shifting the temperature from 30 to 42 °C. Proteins were renatured by dialysis in 250 mM Tris-HCl pH 9.0, 100 mM NaCl, histidinolate, thioredoxin, and/or adenine if required. Glucose or galactose was present at 2%. Hygromycin (Sigma) was present at 200 μg ml−1, to maintain plasmids in yeast. S. cerevisiae strains were transformed using the lithium-acetate method17.

The plasmid for expression of HF-ExoY under control of the arabinose-inducible promoter P_{ara}/(pUM447) was cloned as follows: Primers UM248 and UM254 were used to PCR-amplify exoY from P. aeruginosa PA01 chromosomal DNA. The EcoRI, XbaI digested fragment was used to replace the EcoRI/XbaI fragment from pUM447. pUM449 for arabinose-inducible expression of ExoY-FH was constructed by PCR-amplifying exoY using UM245 and UM250, digestion with EcoRI and XhoI and replacing the EcoRI/XhoI fragment from pUM407. Protein expression levels were low. We, therefore, constructed a plasmid expressing ExoY-FH from L_P1 controlled by the temperature sensitive cI repressor (cI857),
**Affinity purification.** S. cerevisiae cells expressing ExoYIfK11M-TAP [TAP-tag as follows: Calmodulin-binding peptide separated by TEV protease cleavage from the IgG-binding domain (amino acids 80–312 of protein A from *Staphylococcus aureus*, P02976) from pU497 or ExoYIfK11M-HA [3 tandem HA (Human influenza hemagglutinin) tags] from pU498 as mock control were grown in 2 L yeast extract peptone galactose at 30 °C. One step purification using Dynabeads M-270 (Invitrogen) conjugated to rabbit IgG (Sigma, Ref. 19060) were performed according to ref. 53. One half of the methanol/chloroform precipitated protein was analysed by PAGE followed by staining with Bio–Safe Coomassie G-250 (BIO-RAD) followed by silver staining (Pierce). The second half was directly digested by trypsin and analysed by LC–MS/MS analysis at the proteomic facility of the Pasteur Institute (Paris, France). Protein identification and quantitative estimation of the specific enrichment of proteins in the experimental sample as compared with the control.

**LC–MS/MS analysis.** Proteomics analyses were realised at the 3PS proteomics facility, Université Paris Descartes, Sorbonne Paris Cité, Institut Cochin, Paris as previously described55. Briefly: LC–MS protein analysis: peptides from Trypsin–digested extracts were filtered, washed and analysed using a reverse phase C18 column (0.1 cm, 10000 n mole HPLC hyphenated to a Linear Trap Quadrupole–Orbitrap mass spectrometer (all from Thermo)). LTQ MS/MS CID spectra were acquired from up to 20 most abundant ions detected in the Orbitrap MS scan. Proteome discoverer 1.3 (Thermo) with Mascot (matrixscience) was used for protein identification. Separate analyses were compared using the MyPROMS software57.

**Pull-downs and cosedimentation assays.** Actin (MA-L) was converted to Mg-ATP actin and allowed to polymerize to steady state at 20 μM for 2 h at room temperature (Fig. 2a) or prevented from polymerization by the addition of latrunculin A to 30 μM (Fig. 2b), then diluted fivefold in binding buffer (50 mM Na-phosphate pH 8.0, 300 mM NaCl, 25 mM imidazole, 5 mM ATP, 20 mM MgCl2, complete EDTA-free protease inhibitor cocktail (Roche)) and added to 7.5 μL of TAP-tagged ExoY ST (M.W. of 12 μg of ExoY M+) elution buffer (40 mM imidazole). The corresponding beads were collected by centrifugation at 10,000 g for 1 min and left on ice overnight. The unbound or bound fraction and the corresponding inputs were analysed by SDS–PAGE and stained for protein identification. The unbound fraction was concentrated and stored in G-buffer (5 mM Tris-HCl pH 7.8, 0.1 mM CaCl2, 0.2 mM ATP, 5% glycerol) were thawed on ice, then prepared for gel analysis.

**Quantification of cAMP or cGMP synthesis in vitro.** cAMP and cGMP synthesis were measured in 50 μl reactions containing 50 mM Tris–HCl pH 8.0, 7.5 mM MgCl2, 0.5 mg ml−1 BSA, 200 mM NaCl, 1 mM DTT, 2 mM GTP or ATP gapped with 0.1 μCi of [α-32P] GTP, respectively, ExoY and indicated activator. Reactions for Figs 1a and 3a contained in addition 0.02% triton X-100, 10% glycerol, 1 mM DTT, 0.4 mM phenylmethylsulphonyl fluoride). All reactions were measured in 50 mM Tris–HCl pH 8.0, 7.5 mM MgCl2, complete EDTA-free protease inhibitor cocktail (Roche) and added to rabbit IgG (Sigma, Ref. 19060) were performed according to ref. 53. One half of the methanol/chloroform precipitated protein was analysed by PAGE followed by staining with Bio–Safe Coomassie G-250 (BIO-RAD) followed by silver staining (Pierce). The second half was directly digested by trypsin and analysed by LC–MS/MS analysis at the proteomic facility of the Pasteur Institute (Paris, France). Protein identification and quantitative estimation of the specific enrichment of proteins in the experimental sample as compared with the control.

**Actin binding protein Biochem Kit Muscle actin’ with the here specified modifications. Twenty microlitres of a 48 μM actin (MA-L) solution in G’-buffer (5 mM Tris–HCl pH 8.0, 0.2 mM CaCl2, 0.5 mM DTT, 0.2 mM ATP, 5% glycerol) were thawed on ice, then added to 50 μl of 50 mM bis-tris propane pH 9.5 and allowed to sit on ice for 40 min before polymerization was induced according to the protocol. These specific buffer conditions were chosen to allow maximum solubility of ExoY and were shown in our experiment to be compatible with polymerization of actin. Thirty microlitres of polymerized F-actin stock solution were combined with 20 μl of a solution containing 12 μg ExoY-FH in 50 mM bis-tris propane pH 9.5, 270 mM NaCl, 2 mM DTT and 1 × polymerization buffer from which non-soluble aggregates had been removed previously by centrifugation at 54,000 r.p.m. in a TL55 rotor (Beckmann) at 18 °C for 1 h. This mixture as well as controls containing only actin or only ExoY and the corresponding buffers present in the experiment were incubated at room temperature for 30 min and centrifuged at 54,000 r.p.m. for 90 min at 18 °C. Aliquots of supernatant and resuspended pellet fraction corresponding to 15% of the input sample were analysed by SDS–PAGE. To measure the equilibration dissociation constant (Kd) of the ExoY–F–actin complex by co sedimentation assays we used MBP–ExoY/ExoYFK11M-ST and muscle α-actin (MA-L) (Fig. 4c, Supplementary Fig. 6b). MBP–ExoY/ExoYFK11M-ST should provide a reliable estimate of ExoY affinity for F-actin because these constructs perform similarly as ExoY/ExoYFK11M in depolymerization assays. MBP–ExoY/ExoYFK11M-ST allows separation and quantification of unambiguously by densitometry the fraction of the bound toxin at 89.9 kDa from actin at 42 kDa on SDS–PAGE gels, while ExoY ST (M.W. of 43 kDa) was migrating too close to actin (M.W. of 42 kDa). No bundling activity was observed for ExoY in low-speed pelleting assays with F-actin. Three micromolar of F-actin–AP-2 (3 μM Tris–HCl pH 7.4, 1 mM DTT, 5 mM ATP, 2 mM GTP) with or without 6 μM phallolidin (Supplementary Fig. 6b) was mixed for 1 h with increasing amounts of MBP–ExoY/ExoYFK11M–ST (0 to 17.9 μM). The unpolymerized (S, supernatant) and polymerized (P, pellet) fractions were separated by an ultracentrifugation for 30 min at 200,000 g, resolved by 10/15% SDS–PAGE and detected by coomassie blue staining. The ExoY-bound fraction was quantified by densitometry using the ImageJ software and this ExoY-bound concentration normalized by the F-actin concentration was plotted versus ExoY concentration. The following equation was used to fit to the data: Kd is the initial concentration of F-actin, [E0] the total concentration of ExoY in each measurement, and Kd the equilibrium dissociation constant. The fraction R of ExoY bound to F-actin is as follows:

\[ R = \frac{K_d + [E]}{[E] + K_d} \]
Pyrene-actin polymerization and depolymerization assays. Actin polymerization or depolymerization were monitored at 25 °C by the increase or decrease in fluorescence, respectively, of 3–10% (polymerrization) or 50% (depolymerization) pyrene-labeled actin (Py340, 240 nm excitation, 475 nm emission, 17 mW at 10 mW input). Actin-C5 was converted just before the experiments into G-actin-Mg-ATP by adding 1/100 (vol/vol) of (2 mM MgCl2, 20 mM EGTA). Polymerization assays were performed in a final F0 buffer containing (0.1 M KCl, 2 mM MgCl2, 30 mM Tris-HCl pH 7.8, 1 mM TCEP, 1 mM DTT), 5 mM ATP or 2 mM ADP and 4 mM GTP, unless indicated otherwise in figure legends. Polymerization assay with Arp2/3 complex and depolymerization were performed in a final F1 buffer containing (0.1 M KCl, 8 mM MgCl2, 50 mM Tris-HCl pH 7.8, 9 mM TCEP, 1 mM DTT, 0.3 mM (NH4)2SO4, 10 to 15 mM ATP or ADP, and 3 to 5 mM GTP, unless indicated otherwise in figure legends. Fluorescence measurements were carried out in a Safas Xenius model FX (Safas, Monaco) spectrofluorometer, using a 77 mW multi-channel device. Dilution-induced depolymerization assays were performed by quickly diluting 4 to 68 μl of 9 to 13 μM 50% pyrenyl labelled F-actin at steady state into a final volume of 160 μl containing F1 buffer, ATP and the proteins of interest. Different fluorescence intensity levels were used in the two depolymerization assays of Fig. 4b.

Random mutagenesis. Random mutagenesis was performed using the GeneMorph II kit (Agilent) according to the instructions of the manufacturer. PCR was performed using primers 1060 and 1031R on template p1387. Clal/Sall-digested fragments were cloned into plasmid p1182 digested with the same enzymes and transformed into E. coli. Transformants (±4,000) were pooled for plasmid DNA extraction, which was subsequently transformed into S. cerevisiae SC483 to substitute wild-type actin with mutant alleles. This haploid S. cerevisiae strain lacks the chromosomal act-1 gene and containing the F15 gene, which harbours the wild-type act1 gene and a Ura3 marker. Initial transformants were harvested and pooled after 2 days incubation at 30 °C and re-plated on medium containing 5- fluoroorotic acid to select against the plasmid coding for wt actin. Colonies were pooled and kept in frozen stock at -20 °C. A aliquot of the mutant pool stock solution was transformed in batch with plasmid p1654 coding for Myc-ExoY-NanoLuc and plated on SG-agar supplemented with 0.05% glucose. Colonies were picked after 3–4 days and streaked on 5G-agar. Forty of the initial 50 colonies survived under these conditions and were analysed for expression of ExoY by anti-Myc western blots. Plasmid DNA from the 21 clones that did not show significant dense bands on western blots was transformed into E. coli selecting for the kanR marker of the plasmids carrying the actin mutant allele. Sequences of the act-1 mutant alleles were then analysed.

Isolated plasmids (p1688 and p1689) carrying either one of the two distinct mutant alleles that were found (D25Y/D222G and D25N, respectively) as well as p1387 (wild-type actin) were transformed into SC483 to express the plasmid background. The strains obtained after elimination of the wild-type act1 gene (SC690, SC691, and SC489) were used in stress tests to study potential effects of the actin mutant alleles on growth under different conditions and effects on cytotoxicity of ExoY expression.

Light microscopy and quantifications of F-actin content. Hela cells were transiently transfected with AcGFP or ExoYK81M-AcGFP constructs. Transfection efficiency and社科 products were measured by AcGFP fluorescence intensity in stress fibres which was obtained by analysing the bottom three slices after subtraction of background from the nearby cytoplasm. Total amount of F-actin was assessed by the intensity of phallidin staining in the whole cell. Image analyses (region of interest selection as an automatic threshold) were performed using MCID software (Institut Pasteur, France Bio Imaging)59. Statistical analysis was performed using Student’s t-test as significance test, with P<0.001.

For TIRF microscopy, HeLa cells were cultured in DMEM, 5% CO2. Cells were transfected with vinculin-mCherry60 and either GFP-C1 (Clontech, Mountain View, Calif.) or GFP-C2 (Fig. 4d) and were then analysed. Image analyses (region of interest selection as contours of the whole individual cells, intensity measurements, co-localization analyses) were performed using Icy software (Institut Pasteur, France Bio Imaging)59. Statistical analysis was performed using Student’s t-test as significance test, with P<0.001.

Data availability. The authors declare that the data supporting the findings of this study are available within the paper and its Supplementary Information files.

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**Author contributions**

U.M., D.L., C.S., V.O. and L.R. conceived and U.M., D.L., C.S., V.O., A.B., V.D and L.R. designed the experiments. A.B., D.R.-B., C.S., A.N., V.O., L.W., V.D, V.H., S.F., C.M., E.A., L.R. and U.M. performed the experiments and analysed the data. U.M., D.L., C.S. and L.R. wrote the paper. V.O. and V.D. edited the manuscript.

**Additional information**

Supplementary Information accompanies this paper at http://www.nature.com/naturecommunications

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