Tyrosine glycosylation of Rho by Yersinia toxin impairs blastomere cell behaviour in zebrafish embryos

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Yersinia species cause zoonotic infections, including enterocolitis and plague. Here we studied Yersinia ruckeri antifeeding prophage 18 (Afp18), the toxin component of the phage tail-derived protein translocation system Afp, which causes enteric redmouth disease in salmonid fish species. Here we show that microinjection of the glycosyltransferase domain Afp18<sup>G</sup> into zebrafish embryos blocks cytokinesis, actin-dependent motility and cell blebbing, eventually abrogating gastrulation. In zebrafish ZF4 cells, Afp18<sup>G</sup> depolymerizes actin stress fibres by mono-O-GlcNAcylation of RhoA at tyrosine-34; thereby Afp18<sup>G</sup> inhibits RhoA activation by guanine nucleotide exchange factors, and blocks RhoA, but not Rac and Cdc42 downstream signalling. The crystal structure of tyrosine-GlcNAcylated RhoA reveals an open conformation of the effector loop distinct from recently described structures of GDP- or GTP-bound RhoA. Unravelling of the molecular mechanism of the toxin component Afp18 as glycosyltransferase opens new perspectives in studies of phage tail-derived protein translocation systems, which are preserved from archaea to human pathogenic prokaryotes.
ight regulation of cytoskeletal organization and activity enables cells to assemble and function in an organism, but the cytoskeleton is also a target of infectious agents causing severe pathologies. Bacterial virulence factors post-translationally modify key regulators of the cytoskeleton, like the family of Rho GTPases, to disseminate or evade host defense mechanisms. The molecular identification of toxins selectively modifying regulatory components of the cytoskeleton can also be useful to uncover cellular processes, equally important for morphogenesis and disease pathogenesis. Therefore, insights into virulence mechanisms of toxins, particularly those modulating Rho GTPases, may promote the understanding of the control of cellular morphology, motility and cell division.

Rho GTPases control cell shape, polarity and motility by regulation of the actin cytoskeleton. Accordingly, Rho GTPase signalling regulates germ cell migration in the embryo, and epithelial-to-mesenchymal transition initiating neural crest cell migration. Controlled by planar cell polarity signalling, RhoA and its effectors regulate convergence and extension movements of embryonic tissues during gastrulation. Functions of specific Rho GTPases have been analysed during cancer progression, as well as during pathogenic processes on bacterial infection. The use of Rho-specific toxins and the discovery of pharmacological inhibitors of Rho signalling were essential to uncover developmental as well as disease-related mechanisms.

Several Yersinia species are highly pathogenic for humans and animals causing diseases as diverse as plague and enterocolitis. Y. ruckeri is the causative agent of enteric redmouth disease, a generalized septicemia in salmonid fish species. Despite the economic significance of the disease, very little is known about the pathogenesis and even less is reported on molecular virulence mechanisms. Virulence is presumed to be mediated by a phage-derived needle-like particle complex called antifeeding prophage (Afp), which shares key characteristics in common with type VI secretion systems (T6SS), R-type pyocins and the Photorhabdus luminescens virulence cassette PVC. Variants of this gene cassette equip numerous prokaryotes including pathogenic Gram-negative and Gram-positive bacteria but also Archaea. Similar to phage tail-like pyocins and type VI secretion systems, Afps appear to consist of a contractile sheath, an inner tube, baseplate components and tail fibres, but are devoid of a (T6SS), Afps appear to consist of a contractile sheath, an inner tube, baseplate components and tail fibres, but are devoid of a phage head component. The genes Afp1–18 are assumed to encode an ~100-nm contractile phage tail structure that on stimulation contracts a sheath protein complex to eject the inner tube, which penetrates the eukaryotic prey cell. Afp18 appears to be the toxin unit, the ‘warhead’, which is suggested to be enclosed in the core tube of the machinery and injected into eukaryotic host cells. For Serratia entomophila, it was shown that Afp18 is responsible for a toxic antifeeding phenotype when introduced into insect hosts.

To study the Afp18 virulence effecter of Y. ruckeri, we employed the zebrafish embryo as a model, in which cell biological processes during vertebrate development can be visualized at high spatial and temporal resolution. The early cleavage, blastula and gastrula stages of zebrafish development are an excellent model to study individual and collective cellular behaviours. Analyses of infection of zebrafish with Y. ruckeri and other pathogens have provided insights into disease mechanism, as well as function of the innate immune system.

Here we describe the molecular mechanism of the virulence gene product Afp18 from Y. ruckeri. Afp18 mediates toxicity via a glycosyltransferase domain that disrupts RhoA GTPase-dependent actin organization. We find that non-reversible glycosylation at a highly conserved tyrosine residue is responsible for the loss of RhoA activity. Tyrosine glycosylation results in a structural perturbation of the switch I region in RhoA. This inhibits the interaction of RhoA with regulators and downstream signalling effectors, resulting in disturbed actin regulation and abrogates early zebrafish development.

Results

Afp18 gene harbours a putative glycosyltransferase domain. Y. ruckeri Afp18 is a component of the prophage tail-like injection machinery (Afp) and exhibits similarities with Serratia Afp18 in terms of size and amino-terminal architecture, but differs in the carboxyl-terminal toxic domain (Fig. 1a,b, scheme). The toxic domain of Yersinia Afp18 comprises a putative glycosyltransferase domain (Fig. 1b, green coloured region), which exhibits significant sequence similarity to glycosyltransferases from Legionella pneumophila (Lgt1–3), Photorhabdus asymbiotica (PaTox) and clostridial glycosylating toxins, including toxin A and B from Clostridium difficile (Fig. 1b, sequence alignment). All these glycosyltransferases contain a conserved DxD-(aspartic acid–aspartic acid) motif, which is essential for sugar donor substrate binding and, thus, crucial for enzymatic activity. Mutations of this motif result in catalytic defective enzymes.

Afp18G severely affects early zebrafish embryo development. To assess the cellular effects and toxicity of Afp18, we isolated the DNA and cloned the variable carboxyl-terminal fragment (Afp18G; amino acids 1,771–2,123) comprising the putative glycosyltransferase domain. In our studies, we used Y. ruckeri isolated from an infected rainbow trout in Idaho, USA. This strain is identical to Y. ruckeri recently isolated from a wound infection of a 16-year-old male patient in Belgium. We purified the Afp18G protein in E. coli and microinjected the recombinant protein into zebrafish yzoytes at one-cell stage. In addition, we constructed an Afp18G mutant with an exchange of the DxD motif against an enzymatically non-functional NxxN, which we injected as control. Afp18G NxxN injected embryos developed normally and were indistinguishable from non-injected or buffer injected control embryos. Afp18G-injected embryos performed the first three to four cell divisions with morphologically visible cleavage planes between the dividing blastomeres (Fig. 1c, 16-cell stage, arrows), albeit progress of development was delayed compared with controls. At the 256-cell stage, 2.5 h post fertilization (h.p.f.), control embryos showed normal development of the blastoderm positioned on top of the large vegetal yolk cell. In contrast, Afp18G-injected embryos failed to establish the typical multilayered organization of the blastoderm and large sections of the blastoderm were devoid of morphologically discernible cell boundaries (Fig. 1c, 256-cell stage, arrow). About 1 h later, at the onset of gastrulation, control embryos initiated epiboly, a coordinated cell movement, in which the static blastomeres became motile and spread vegetalward to cover the yolk cell. In contrast, Afp18G-injected embryo did not initiate epiboly, the blastoderm disrupted and in most severe cases the yolk cell and (or) blastomeres lysed and embryos completely disintegrated (Fig. 1c, 30% epiboly, arrowhead and asterisk, respectively). These data reveal that the glycosyltransferase domain of Afp18 is crucial for its severe toxic effect on early zebrafish development.

Dose-dependent effects of Afp18G on embryo development. To rule out contribution of effects by contaminants from the E. coli-derived protein preparation, we microinjected mRNA encoding the Afp18G protein. Dilution series of Afp18G encoding mRNA facilitated to determine effects of a wider range of toxin dosage. We injected 0.1–100 pg in vitro transcribed Afp18G or
**Figure 1** | *Yersinia ruckeri* antifeeding prophage tail (**Afp**) translocation system and effects of the glycosyltransferase domain of **Afp18**.

(a) Organization of **Afp** genes from *Yersinia ruckeri* and *Serratia entomophila* with their predicted and ascribed protein domains. The toxin units are shown in red. Bottom: pairwise genomic gene analysis of aligned **Afp** gene clusters using mVISTA (http://genome.lbl.gov/vista). Indicated is the average per cent of nucleotide identity within a window of 20 nucleotides. Nucleotide identity > 70% is shaded in red. (b) Architecture of *Y. ruckeri* **Afp18** and similarity to the virulence gene product **Afp18** from *S. entomophila*. Amino-acid sequence alignment of the region surrounding the DxD motif (marked) of different toxin glycosyltransferases. Secondary structure elements are deduced from the crystal structure of PaTox (pdb code 4MIX). Accession numbers are the following: *P. asymbiotica* PaTox (PaGT, accession number CT8K69), *Legionella pneumophila* glucosyltransferase 1 (Lgt1, accession number Q5ZVS2), *Clostridium difficile* toxin B (TcdB, accession number P18177), *C. sordelli* lethal toxin (TcsL, accession number Q46342), *C. novyi* α-toxin (TcnA, accession number Q46149). Alignment was prepared using ClustalW and rendered using ESPript 3.0 (www.espript.ibcp.fr). Identical residues are boxed and shown in red, similar residues are boxed in yellow. (c) Live images of non-injected, PBS buffer control, **Afp18**G NNN and **Afp18**G protein (each 3 μM, 1 nl injection) injected zebrafish embryos at indicated developmental stages, 16-cell (1.5 h.p.f.), 256-cell (2.5 h.p.f.) and 30% epiboly (4.7 h.p.f.). Arrows mark blastomere cleavage furrows at 16-cell and 256-cell, embryos are oriented animal to the top. At the 16-cell stage, **Afp18**G-injected embryo blastomeres show cleavage planes, while blastomeres fail to establish cell boundaries at the 256-cell stage. Control embryos at 30% epiboly develop normally compared with non-injected WT, while **Afp18**G-injected embryos show disrupted blastoderm (arrowhead) or disintegrate completely (asterisk). Scale bar, 200 μm.

**Afp18**G NNN mRNA per embryo, or as control **GFP** mRNA. Morphological phenotypes were documented at the 1,000-cell stage (3.3 h.p.f.; Fig. 2a, b quantitative analysis; Supplementary Fig. 1). Embryos injected with 0.1 pg **Afp18**G mRNA did not develop significantly different from control embryos. About 30% of the embryos injected with 0.5 pg **Afp18**G mRNA developed a disintegrated blastoderm, with irregular cell shape, local loss of blastomere boundaries and abnormal cell sizes (arrowhead in Fig. 2c, 0.5 pg). Injection of 1 pg **Afp18**G mRNA per embryo affected cell morphology in all analysed embryos. Frequently,
Figure 2 | Afp18G disturbs zebrafish early development. (a) Live images of non-injected, GFP, Afp18G NxN mRNA (each 100 pg per embryo), or different amounts (0.1–100 pg per embryo) of Afp18G mRNA-injected embryos at 1,000-cell stage (3 h.p.f.). Embryos oriented animal to the top. Scale bar, 500 μm. (b) Quantification of the blastoderm phenotype of control and Afp18G-injected embryos shown in a at 1,000-cell stage (non-injected, n = 41; GFP mRNA, n = 37; Afp18G NxN mRNA, n = 52; 0.1 pg Afp18G, n = 35; 0.5 pg Afp18G, n = 45; 1 pg Afp18G, n = 60; 10 pg Afp18G, n = 62; 100 pg Afp18G, n = 60). Only values for 100 pg per embryo injected GFP and Afp18G NxN mRNA are shown. Developing live embryos were classified into categories ‘normal’ (WT like), ‘disrupted blastoderm’ (cellular structure of blastoderm abnormal) or ‘disintegrated embryos’ (blastoderm and (or) yolk cell lysed). The distribution of phenotypes was analysed for significant differences using Fisher exact probability test, revealing significant differences (*P values < 0.0001) between Afp18G NxN control and Afp18G samples. (c) Optical image planes of live blastoderm regions at 1,000-cell stage (3 h.p.f.) of non-injected, GFP, Afp18G NxN or Afp18G mRNA-injected embryos (pg per embryo) oriented animal to the top. Control embryos show normal development of blastomeres, while the blastoderm progressively loses cellular integrity with increasing amounts of Afp18G mRNA injected. Arrowheads mark the disrupted regions without visible blastomere boundaries, abnormal sized and irregular shaped cells. Asterisks mark detached blastomeres and disintegrated blastoderm. Scale bar, 100 μm. (d) Live images of control, full-length Afp18 and full-length Afp18 NxN mRNA (each 50 pg per embryo) injected zebrafish embryos. Afp18G-injected embryos disintegrate at 1,000-cell stage (3 h.p.f., upper row, embryos are oriented animal to the top) and degrade early in gastrulation (lower row 40% epiboly, 5 h.p.f.). Scale bar, 500 μm. For five different concentrations each of Afp18 (non-injected n = 80; 2.5 pg n = 34; 5 pg n = 50; 12.5 pg n = 65; 25 pg n = 85; 50 pg n = 38) and Afp18 NxN (2.5 pg n = 43; 5 pg n = 58; 12.5 pg n = 50; 25 pg n = 76; 50 pg n = 23) mRNA injections, the bar graph shows percentage of embryos, which at 5 h.p.f. develop like WT controls or degrade.
larger cells detached from the blastoderm and abnormal vesicles were formed (arrowhead in Fig. 2c). When 10 or 100 pg Afp18\(^G\) mRNA were injected per embryo, cell morphology was severely affected, tissue integrity progressively lost during gastrulation and blastoderm frequently disintegrated (Fig. 2c and Supplementary Fig. 1a). In contrast, microinjection of mRNA encoding the glycosyltransferase-deficient mutant Afp18\(^G\) NxN had no effect on embryonic development, revealing that the effects were indeed caused by the glycosyltransferase activity. Given that the effects of Afp18\(^G\) on cellular functions cannot be analysed when embryos disintegrate early, we chose to inject 0.5 pg Afp18\(^G\) mRNA for further analyses.

**The G domain is the major pathogenicity determinant in Afp18.**

Next we wanted to clarify whether parts of Afp18 other than the glycosyltransferase (G) domain affect zebrafish embryo development. Therefore, we injected mRNA encoding the full-length Afp18 protein, or the enzymatically non-functional Afp18 NxN mutant, and analysed embryos and early larvae to identify potential morphological alterations in embryogenesis. Full-length Afp18 caused severely degraded embryos during early gastrulation (40% epiboly; Fig. 2d), while Afp18\(^G\) NxN injected embryos developed indistinguishably from non-injected wild-type (WT) control embryos (Fig. 2d). These data revealed that the glycosyltransferase domain is the Afp18 protein domain mediating toxicity in this assay.

**Afp18\(^G\) disrupts the actin cytoskeleton.** To analyse the in vivo cellular components affected by the Afp18\(^G\) toxin, we co-injected mRNAs encoding Lifeact-GFP (green fluorescent protein) to fluorescently label the actin cytoskeleton and histone H2B-dsRed to label nuclei in living embryos. On Afp18\(^G\) mRNA injection, blastomeres were severely enlarged (Fig. 3a, asterisk). In addition, blastomeres frequently contained two or more nuclei. We assumed that these Afp18\(^G\)-induced morphological alterations were caused by deregulation of F-actin cytoskeleton organization. The Lifeact-GFP signal suggested a reduced amount of polymerized actin in Afp18\(^G\)-expressing embryonic cells (Fig. 3a, Supplementary Movie 1). To analyse the effects of Afp18\(^G\) on the actin cytoskeleton in more detail, we introduced recombinant 6xHis-tagged glycosyltransferase domain protein into zebrafish ZF4 cells (embryo-derived fibroblast-like cell line)\(^3\), using protective antigen (PA; the binding and translocation component of anthrax toxin) as a delivery system\(^34\), and stained filamentous actin with TRITC-phalloidin. Cells to which the glycosyltransferase-deficient NxN-mutant was delivered (Fig. 3b, middle panel) had an actin cytoskeleton stained filamentous actin with TRITC-phalloidin. Cells to which Afp18\(^G\) was delivered (Fig. 3b, bottom right panel) and finally rounded up (Fig. 3b top right panel). Live imaging of ZF4 cells transfected with GFP-actin revealed a complete disassembly of the actin cytoskeleton after delivery of Afp18\(^G\) (Fig. 3c, time series bottom row; top row
Afp18G NxN-treated control cell). The actin-depolymerizing effect was remarkably rapid. During 60 min of incubation, actin fibres disappeared and 2Z4 cells collapsed. Comparable effects were observed using human HeLa cells (Supplementary Fig. 3a). Thus, the glycosyltransferase activity of Afp18 appears to severely affect the regulation of cellular actin.

**Afp18G affects cytokinesis in early development.** We examined whether the completion step of cell division, involving coordinated actin rearrangement, was affected by Afp18. We found that cytokinesis proceeded normally in Afp18G NxNexpressing control embryos (Fig. 4a, upper row, arrow; Supplementary Movie 1). In contrast, cytokinesis including the assembly of the actomyosin ring and formation of the cleavage furrow was severely impaired in blastomeres of Afp18G mRNA-injected embryos, resulting in cells frequently containing more than one nucleus (Fig. 4a, lower row, arrow; Supplementary Movie 1). We evaluated cortical and cytoplasmic deposition of F-actin via measurement of the integrated density of Lifeact-GFP fluorescence (Fig. 4b). The ratio of cytoplasmic versus cortical F-actin localization at three developmental time points (sphere, 30% epiboly, 50% epiboly) was almost constant in blastomeres of Afp18G NxN control injected embryos, when measured in 30 min time windows. In contrast, cortical actin was significantly enriched in blastomeres of Afp18G mRNA-injected embryos, indicating a deregulation of the dynamic organization of the actin cytoskeleton. However, the cell cycle phases involving proper assembly and organization of microtubule-based mitotic spindle progressed normally. Time series of Lifeact-GFP and H2B-dsRed labelled embryos revealed the accurate composition of the metaphase, with the sister chromatids moved to the opposite spindle poles for both, Afp18G NxN (Fig. 4a, upper row) and Afp18G (Fig. 4a lower row) mRNA-injected embryos. In summary, Afp18G seemed to selectively affect actin-dependent filament organization and dynamics during cytokinesis, while microtubule dynamics and karyokinesis appeared to progress normally.

**Bleb formation is impaired by Afp18G.** The organization of the cortical actin network controls cell membrane protrusions, and, thus, has a strong influence on cell motility. It was shown that spherical protrusions, called blebs, are formed dynamically at the membrane of migrating cells. We scored bleb formation of blastomeres of Afp18G and Afp18G NxN mRNA-injected embryos at the onset of epiboly. Fig. 5a and Supplementary Movie 2 show a time series of a forming bleb documented by differential
interference contrast transmitted light (upper row, black arrowhead) and fluorescence microscopy of Lifeact-GFP (lower row, white arrowhead) of a control injected embryo (upper two rows). The three phases of a bleb life cycle—initiation (after 10 s), expansion (up to 30 s) and retraction (40 to 70 s)—were clearly visible. Initially, blebs form and grow devoid of actin, while during retraction fluorescently labelled Lifeact-GFP-actin signal appeared slightly enhanced. Blebbing was severely reduced in numbers but also in bleb size in Afp18G mRNA-injected embryos (Fig. 5a lower two rows), as quantified by bleb counts (Fig. 5a graph). In addition, we analysed other actin-driven protrusion behaviours of blastomeres, excluding blebbing. Time series of both Afp18G and Afp18G NxN mRNA-injected embryos showed blastomeres, which form lamellipodia driven by actin remodelling (marked by black and white arrows in differential interference contrast and fluorescence images, respectively; Fig. 5b and Supplementary Movie 2). Quantification revealed that blastomeres did not significantly differ in lamellipodia number (membrane protrusions with actin remodelling—see graph in Fig. 5b) when Afp18G and Afp18G NxN expressing embryos were compared.

Blebs have been reported to be primarily controlled by RhoA and its effector ROCK-1 \(^{37,38}\). In contrast, Rac and Cdc42, which
Figure 6 | Afp18G selectively modifies RhoA using UDP-GlcNAC. (a) Donor substrate specificity of Afp18G determined by UDP-glycosidase activity. Percentage of hydrolysed UDP-[14C]sugars was determined by PEI thin-layer chromatography and autoradiography after incubation for 10 min at 30°C. Data are representative of three independent experiments. (b) Autoradiography of the SDS–PAGE from cell lysate incubated with Afp18G and indicated radiolabeled UDP-sugars. Western blot of RhoA (right panel) showed similar electrophoretic mobility as radiolabelled proteins. (c) Time course of in vitro GlcNAcylation of RhoA, Rac1 and Cdc42 by Afp18G (1 nM). Inserts show representative autoradiograms (upper panel) and Coomassie-stained SDS–PAGE (bottom panel). Error bars indicate s.e.m.’s of three technical replicates. (d) Extracted ion chromatograms of thermolysin-digested GST-RhoA, GST-Rac1 and Cdc42 treated with Afp18G (lower chromatogram) or untreated control (upper chromatogram). The molecular mass [M + 2H]^2+ of the switch I peptides (RhoA: 26SKDFPHEYVTPT37; Rac1: 24TTNAFPGEYPT35; Cdc42: 24TTNFPSFEPYPT35) are indicated. Afp18G-modified GTPases (lower chromatogram) show a mass shift of 203 Da revealing a modification with a single N-acetylglucosamine (shifted blue curves). In comparison with Rac1 and Cdc42, RhoA was modified more efficiently. (e) The DxD motif of Afp18 is crucial for GlcNAcylation of RhoA. Recombinant RhoA was incubated in the presence UDP-GlcNAz or UDP-[14C]GlcNAc with Afp18G, Afp18G NxN or without toxin. Modified proteins were analysed by click chemistry using biotin alkyne and western blotting and autoradiography, respectively. Anti-RhoA served as input control.

Af18G utilize UDP-N-acetylglucosamine to modify Rho GTPases.

To identify the cellular targets of Afp18 in zebrafish, we elucidated the sugar donor for this reaction by enzyme-catalysed UDP-[14C]sugar hydrolysis and found that Afp18G efficiently hydrolysed UDP-[14C]GlcNAC (Fig. 6a). Using UDP-[14C]GlcNAC in a glycosylation reaction with Afp18G and cell lysate, we identified proteins with an electrophoretic mobility corresponding to 23 kDa, which were labelled with [14C]-GlcNAC and migrated similarly to the GTPase RhoA (Fig. 6b). As Rho GTPases are known regulators of the actin cytoskeleton and putative substrate candidates, we applied RhoA, Rac1 and Cdc42 to an in vitro glycosylation reaction with 1 nM Afp18G and obtained a strong modification of RhoA. Signals of Rac1 and Cdc42 were hardly visible under these conditions (Fig. 6c). When we applied higher amounts of Afp18G (100 nM), we could also observe the glycosylation of RhoB, RhoC, Rac2 and Rac3, and Cdc42 (Supplementary Fig. 3b). Other subfamily members of the Rho family were not modified and also Ras proteins did not serve as substrates. Using mass spectrometric analysis of Afp18G glycosylated RhoA, Rac1 and Cdc42, we could confirm that these GTPases were modified by a single covalently attached N-acetyhexosamine (HexNAc) moiety, which resulted in a mass increase of 203 Da in the corresponding switch I peptides (Fig. 6d). Again, RhoA was modified most efficiently among these GTPases. Thus, RhoA may be the primary target of Afp18G and might explain the previous results obtained in RhoA-, Rac1- and Cdc42-mediated actin dynamic analysis in zebrafish embryos and ZF4 cells. To prove that the NxN mutant of Afp18 is inactive and not able to glycosylate RhoA, we used UDP-GlcNAz as donor in a click chemistry reaction with biotin alkyne and radiolabelled UDP-[14C]GlcNAC in a glycosylation reaction and show that the mutant indeed is deficient in glycosyltransferase activity (Fig. 6e).

Furthermore, we analysed the nucleotide status of RhoA which is increase actin-dependent lamellipodia formation, inhibit bleb formation and amoeboid migration. Therefore, our in vivo data suggested that Afp18G predominantly targeted RhoA, whereas Rac1 and Cdc42 might be less affected. To determine whether Afp18 may co-localize with RhoA, we used DNA vector injection to generate mosaic embryos in which individual cells were expressing enzyme-deficient EGFP-tagged Afp18G NxxN and RHOA. Anti-EGFP and anti-RHOA immunofluorescence revealed that EGFP-Afp18G NxxN appeared to target the cell membrane, and evaluation of cell lines of fluorescent profiles shows co-localization with RHOA (Supplementary Fig. 2).
glycosylated by Afp18 and found that Afp18 preferentially modified GTP (GTP\(^\gamma\)S)-bound RhoA in comparison to GDP-bound or nucleotide-free RhoA (Supplementary Fig. 4a). GlcNAcylated RhoA is observed in an inhibited conformation.

To identify the site of modification and gain insights into the structural alteration caused by GlcNAcylation of RhoA, we crystallized Afp18-modified RhoA in the presence of magnesium and GDP and solved the X-ray structure at 2.0 Å resolution (Fig. 7a, Table 1). In the RhoA structure, we could clearly assign additional electron density at the hydroxyl group of tyrosine-34 to an attached N-acetylglucosamine moiety (Fig. 7b, Supplementary Fig. 3c). Interestingly, despite the presence of magnesium in the crystallization conditions, the structure revealed an unusual GDP-bound but magnesium-free conformation. The overall fold of the crystal structure of GlcNAcylated RhoA is very similar to known structures of Rho GTPases. However, the switch regions, especially the switch I region, adopt a conformation with tyrosine-34 positioned away from the nucleotide binding pocket, resulting in a structure distinct from the structures of RhoA bound to GDP\(^\gamma\)S or GTP\(^\gamma\)S, which is generally not compatible with effector and regulator interaction (Fig. 7c).

Tyrosine-34 is located within the effector loop region conserved in all Rho family GTPases. In \textit{in vitro} glycosylation experiments with Afp18, followed by tandem mass spectrometric (LC–MS–MS) analyses, we confirmed that also Cdc42 was GlcNAcylated at tyrosine-32 (Supplementary Fig. 3d). Furthermore, site-directed mutagenesis and \textit{in vitro} glycosylation experiments with Y32(34)F mutants of RhoA, Rac1 and Cdc42 confirmed switch I tyrosine-32(34) as the acceptor site of modification (Fig. 7d, Supplementary Fig. 3e). No other hydroxyl-containing amino acid (threonine or serine) or recently discovered glycosyl acceptor residues as tryptophan or arginine were able to substitute tyrosine in RhoA as an acceptor residue for glycosylation (Fig. 7d).

Afp18 forms deglycosylation-resistant \(\alpha\)-glycosidic bonds. The defined electron density of the sugar attached to the hydroxyl group of tyrosine-34 revealed the \(\alpha\)-anomeric configuration of the glycosidic bond (Fig. 7b). This finding implies that the glycosylation mechanism proceeds under retention of the stereochemistry of D-\(\alpha\)-GlcNAc. Thus, Afp18 can be grouped into the family of retaining glycosyltransferases. The stereochemistry of the glycosidic bond has most likely no influence on the functional consequences of Afp18-mediated glycosylation, but might have an influence on the stability of the glycoside inside the

**Figure 7 | Structural consequences of RhoA GlcNAcylation at tyrosine-34.** (a) Crystal structure of glycosylated RhoA at tyrosine-34. Switch I and switch II regions are highlighted in blue. GDP is shown as sticks and balls in black. The GlcNAc moiety attached to tyrosine-34 is shown as sticks and balls in yellow. (b) Electron density map of a section of 2F\(_{o}\)−F\(_{c}\) protein (grey), contoured at a level of 1\(\sigma\), showing GlcNAc moiety attached to tyrosine-34 of RhoA in the alpha configuration of the glycosidic bond (marked). Additional residues of the switch I region were omitted for clarity. (c) Superposition of the effector loops of GlcNAcylated RhoA with structures of non-glycosylated active GTP\(^\gamma\)S-bound RhoA (pdb code 1A2B)\(^\text{42}\) and inactive GDP-bound RhoA (pdb code 1FTN)\(^\text{41}\). The switch I and II regions of GlcNAc-modified RhoA adopt distinct open conformations. (d) Autoradiograms and Coomassie stainings of Afp18\(^\text{G}\)-catalysed \textit{in vitro} \(\text{\textsuperscript{14}}\)
C-GlcNAcylation of WT GST-RhoA and the indicated mutants. (e) Cytoplasmic OGA is unable to revert GlcNAcylation of Rho. RhoA was radioactively preglycosylated by Afp18\(^\text{G}\) and applied to a deglycosylation reaction with GST-OGA. TAB1, preglycosylated by OGT, served as positive control. Autoradiographs (top panels) of \(\text{\textsuperscript{14}}\)
C-GlcNAcylated RhoA and GlcNAcylated TAB1 are shown. Coomassie staining of RhoA, TAB1 and GST-OGA are shown as input controls.
host cytoplasm. Only one enzyme, namely O-GlcNAcase (OGA) exists in eukaryotic cytoplasm, which is able to remove mono-O-
GlcNAc moieties from proteins. OGA predominantly cleaves
sugars attached in the β-configuration. To clarify whether the
glycosidic bond on RhoA was resistant to hydrolysis of OGA, we
tested 14C-GlcNAcylated RhoA in a deglycosylation reaction. As
a control protein, we used TGF-beta-activated kinase 1 (TAB1)
(OGT), which was efficiently deglycosylated by OGA (Fig. 7e).

RhoA GlcNAcylation blocks regulator and effector interaction.
To elucidate the molecular consequences of tyrosine-34
GlcNAcylation of RhoA, we analysed the nucleotide binding
site-specifically at a switch I tyrosine residue. RhoA modification blocked leukaemia-associated RhoGEF
mant-GppNHp, a non-hydrolysable GTP analogue, (Fig. 8a),
and nucleotide exchange of RhoA by mant-GDP fluorescence
GlcNAcylation of RhoA, we analysed the nucleotide binding
to elucidate the molecular consequences of tyrosine-34
Afp18 is a mono-

| Table 1 | Data collection, phasing and refinement statistics for RhoA Y34(GlcNAc) |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| **RhoA Y34(GlcNAc)** | **RhoA Y34(GlcNAc)** | **RhoA Y34(GlcNAc)** | **RhoA Y34(GlcNAc)** | **RhoA Y34(GlcNAc)** | **RhoA Y34(GlcNAc)** | **RhoA Y34(GlcNAc)** |
| **Data collection** | **Space group** | **P4 2 1 2** | **Cell dimensions** | **α, β, γ (Å)** | **91.39, 91.39, 56.62** | **Resolution (Å)** | **50-2.0 (2.07-2.00)** |
| | **R̂merge** | **0.108 (0.319)** | **I/σI** | **21.3 (5.5)** | **Completeness (%)** | **100.0 (100.0)** | **Redundancy** | **5.8 (5.6)** |
| **Refinement** | **Resolution (Å)** | **50-2.0** | **No. of reflections** | **185,873** | **No. of unique reflections** | **31,941** | **No. of atoms** | **1,399** |
| | **Protein** | **14** | **GDP** | **28** | **Sulfate** | **5** | **Water** | **80** |
| | **GlcNAc** | **18.65** | **GDP** | **26.32** | **Sulfate** | **24.75** | **Water** | **22.75** |
| | **r.m.s deviations** | **0.108 (0.319)** | **Bond lengths (Å)** | **0.013** | **Bond angles (°)** | **1.67** |

Abbreviation: r.m.s., root mean squared. For each data set one crystal was measured. *Values in parentheses are for highest-resolution shell.

Afpl8G-phenotype suppression by RhoA overexpression. To
determine whether Afpl8G predominantly acts through the
glycosylation of RhoA in vivo, we tried to rescue the toxin
phenotype by co-injection of mRNA encoding human WT
RHOA or the non-glycosylatable mutant version RHOA Y34F.
Zebrafish RhoA as well as other Rho GTPases share over 95%
sequence conservation with their human homologues16. Embryos
injected with human RHOA or RHOA Y34F mRNA (up to
50 pg per embryo) alone developed normal, indistinguishable
from non-injected or GFP mRNA-injected control embryos
(Supplementary Fig. 5a). We co-injected RHOA or RHOA Y34F
mRNA with 0.5 pg Afpl8G mRNA and found the disrupted
blastoderm phenotype rescued to a large extent, with more
embryos developing normally compared with Afpl8G mRNA-
j ected embryos (Fig. 8d and quantification Fig. 8e). Taken
together, both human RHOA and RHOA Y34F overexpression
were able to rescue development of early embryos from Afpl8G
toxicity.

Discussion
Here we unravelled the mode of action of the toxic
effector Afpl8 of the prophage tail-like protein translocation machinery Afp from Y. ruckeri, which is the causative agent of enteric redmouth
disease in salmonid fish species. We employed Afpl8 for studies
in zebrafish embryos, which have been shown to be a highly
sensitive fish model for effector and toxin analyses26–28,47.

We observed that microinjection or expression of Afpl8 in
zebrafish early embryos abrogated development, and embryos
died before gastrulation was completed. The glycosyltransferase-
deficient mutant (Afpl8 Nxn) showed no developmental defects
unravelling the glycosyltransferase domain as a major toxic
determinant. Even at low expression of the glycosyltransferase
domain Afpl8G alone (0.1 pg Afpl8G mRNA), cytokinesis was

only blocked by the addition of UDP-GlcNAc. Thus, RhoA
GlcNAcylation by Afp18 prevented the activation step of the
GT-Pase. In addition, the interaction of RhoA to its GT-Pase-
activating protein p50RhoGAP was impaired by Afp18G-
mediated glycosylation of tyrosine-34 (Supplementary Fig. 4c).

Next, we analysed the interaction of Rho, Rac1 and Cdc42 with
its downstream effectors Rho kinase α (ROCKII)- and p21-
associated kinase (PAK) and used zebrafish (ZF4) cells in an
effector pull-down assay using ROCKII- and PAK-coupled beads
(Fig. 8c). To activate Rho GTPases independently of intracellular
GEEs, we pretreated cells with cytotoxic necrotizing factor 1
(CNF1), a toxin that activates Rho GTPases constitutively by
deamidation46. Subsequent cell intoxication with Afp18G totally
blocked RhoA interaction with ROCKII, whereas RhoA could
efficiently be precipitated from cells treated with the
glycosyltransferase-deficient mutant Afp18G Nxn. Toxins B
from Clostridium difficile (TcdB), which glycosylates threonine-
37 in RhoA and thereby inhibits Rho effector interaction, served
as a control. Impaired effector interaction catalysed by Afp18G
was also observed for Rhotekin, an effector of RhoA, in human
HeLa cells (Supplementary Fig. 4d). Intriguingly, the interaction
of Rac1 or Cdc42 with their effector PAK could not be blocked by
Afpl8-mediated GlcNAcylation, whereas TcdB treatment of ZF4
cells efficiently prevented PAK interaction (Fig. 8c). This is
consistent with our findings in time-lapse microscopy of Afpl8-
intoxicated ZF4 cells, which showed a rapid degradation of
filamentous stress fibres but a persistence of membrane dynamics
like membrane ruffling and filopodia formation, which are
regulated by Rac1 and Cdc42, respectively (Fig. 3c, 
Supplementary Movie 3). It seems that Afp18 specifically
inactivates RhoA signalling, but less signalling of Rac or Cdc42.
severely affected and multicellular cells were formed. While microtubule-dependent processes like karyokinesis, including metaphase formation and chromatid segregation, progressed normally, actin filament dependent processes (for example, cytokinesis) were blocked. This finding became even more obvious by cell behaviour defects observed during gastrulation, when cells become motile. Afp18G-expressing blastomeres contained significantly less cytoplasmic filamentous actin compared to controls.

Notably, the Yersinia effector-expressing blastomeres had severely reduced blebbing activity. Membrane blebbing depends on actomyosin and is essential for amoeboid cell migration. Accordingly, the combined reduction of blebbing and protrusive activity effectively blocked cell movements and progress of gastrulation, and finally abrogated embryo development. Deducing from the specific effects of Afp18 on the actin cytoskeleton (for example, inhibition of blebbing), we proposed that the observed defects of zebrafish embryo severely affected and multicellular cells were formed. While microtubule-dependent processes like karyokinesis, including metaphase formation and chromatid segregation, progressed normally, actin filament dependent processes (for example, cytokinesis) were blocked. This finding became even more obvious by cell behaviour defects observed during gastrulation, when cells become motile. Afp18G-expressing blastomeres contained significantly less cytoplasmic filamentous actin compared to controls.

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development depend on an action of Afp18G on the GTP-binding protein RhoA. This hypothesis was supported by numerous previous reports, showing that RhoA and its effector ROCK are essential for bleb formation and for amoeboid migration39,40. Notably, Rac and Cdc42, which are also master regulators of the actin cytoskeleton and crucially involved in control of lamellipodia-dependent mesenchymal migration, were shown to inhibit bleb formation and amoeboid migration in various model systems39,40. Moreover, a discrete pool of active RhoA is reportedly essential for the local assembly of the contractile actomyosin ring and for cytokinesis49. In contrast, in numerous studies, it was shown that RhoB, RhoC, Rac1 and Cdc42 are not essential and dispensable for cytokinesis49-52. Moreover, our finding that the overexpression of human RhoA and the non-glycosylatable mutant RhoA Y34F resulted in the suppression of the toxic phenotype was in line with the view that RhoA plays a predominant role in Afp18-induced developmental defects. This hypothesis was strongly supported by the finding that the effector protein Afp18 directly targets RhoA by GlcNAcylation.

We determined the crystal structure of Afp18-GlcNAcylated RhoA and identified the covalent sugar modification at tyrosine-34. RhoA modified at this site revealed an opened conformation of the switch I region, distinct from structures of RhoA bound to GDP41 or GTP42. It was shown that the switch I region of RhoA and especially tyrosine-34 is located in the interface of functional complexes of RhoA and its GEFs or its effectors53. We assume that steric constraints of the attached GlcNAc-residue results in the distorted conformation, which is incompatible with regulator and effector interaction. This assumption was underlined by the finding that the RhoGEF LARG, p50RhoGAP and the effectors ROCK and Rhotekin were unable to interact with Afp18-modified RhoA. Further experiments revealed that higher concentrations of Afp18 also GlcNAcylated Cdc42 and Rac in vitro. The modification of tyrosine-32 was verified by impaired glycosylation of site-directed mutants and in the case of Cdc42 by mass spectrometric analysis. Remarkably, we found that after the expression of Afp18 in target cells, which blocked RhoA functions and inhibited the interaction of the GTPase with its effectors, Rac1 and Cdc42 were still able to interact with their effector PAK. This suggests that in vivo Afp18 is specific for RhoA, a finding which is in agreement with our results on the toxic effects of Afp18 in zebrafish embryos and the time-resolved morphological effects on the actin cytoskeleton in zebrafish (ZF4) cells. However, the overexpression of RhoA or the non-glycosylatable mutant RhoA Y34F did not totally rescue Afp18-dependent developmental defects. Therefore, we cannot entirely exclude that Afp18G modulates other Rho GTPases in vivo as well, which then may contribute to the developmental phenotype.

GlcNAcylation of tyrosine-34(32) of Rho GTPases was recently reported for PaTox, an exotoxin from entomopathogenic Photorhabdus asymbiotica7. In this study, PaTox-induced modification of RhoA was analysed by 1H-NMR spectroscopy. Our crystallization data extend these findings and show a conformation of the switch I region, which was not obvious from NMR data. Interestingly, both enzymes Afp18 and PaTox modify preferentially the active GTP-bound state of GTPases. While PaTox harbour an additional deamidase domain that deamidates heterotrimeric G proteins and is suggested to be involved in Rho activation, Afp18 possesses no typical deamidation domain. It remains to be clarified whether and how Afp18 causes activation of RhoA. Tyrosine-32(34) in Rho GTPases is also known to be modified by phosphorylation43,45, nitration56 and adenylation (AMPylation)57 indicating a pivotal role of this residue in the endogenous regulation of Rho GTPase signalling. In contrast to well-known modifications of serine or threonine residues, glycosylation of a tyrosine residue has not been described in mechanistic detail.

Recently, several novel mono-O-glycosyltransferases were identified, which perform unusual amino-acid modifications in proteins, targeting arginine44,45, tryptophan43 and tyrosine42. Most of these enzymes derive from bacterial species and function as highly active bacterial toxins or effectors in eukaryotes. Site-directed mutagenesis of tyrosine-34 of RhoA to serine, threonine, tryptophan and arginine inhibited modification by the Yersinia glycosyltransferase showing that Afp18 is highly specific and accepts only tyrosine as an acceptor amino acid. Moreover, we analysed the stability of the covalent attached sugar by an in vitro deglycosylation assay with OGA that is the only known O-GlcNAcase in the eukaryotic host cytoplasm. In agreement with the crystal structure obtained, showing that GlcNAc is linked to RhoA in an α-anomeric configuration, OGA, which specifically cleaves β-glycosidic GlcNAc moieties, was not able to remove the sugar from RhoA. Thus, eukaryotic host cells seem not to be able to cope and revert these unusual post-translational modification making Afp18 a highly efficient toxin and consequently, resistance against Yersinia Afp might not be possible by simply upregulating OGA expression in the prey cell.

Our knowledge about Afp prophage tail-like translocation machineries is still in its infancy. Recently, the Afp translocation apparatus of Serratia entomophila, the closest orthologue of the Yersinia Afp, has been described and first structural insights were obtained by negative stain electron tomography20,22. Afp from Y. ruckeri contains all genes required to build the contractile prophase tail injection apparatus and harbours the toxin effector Afp18, which is suggested to be caged inside the tail tube and injected into host cell cytoplasm.53. Remarkably, from genome sequence analyses, a broad distribution of this system in prokaryotes including archaea has been proposed21. Afp-related systems are the Photorhabdus luminescens virulence cassette PVC and the phage tail-like R-type pyocins (also called bacteriocins), which share similarity with the type VI secretion system (T6SS). Effector proteins of phage tail-derived secretion systems have diverse functions both in competition with other prokaryote predators (antibacterial activity) and in interaction with eukaryotic host cells. In contrast to common type VI secretion systems, the Afp system is probably released as preformed injection ‘torpedos’ and is not associated to the bacterial donor cell20,58. Thereby, the system is extremely efficient; only 500 particles of S. entomophila Afp have been reported to kill the New Zealand grass grub (Costelytra zealandica) host23. Recent studies unravelled the mode of action of several T6SS effectors59-62. However, to date, the molecular mechanism of Afp effectors remained enigmatic. Thus, our findings that Y. ruckeri Afp18 harbour a glycosyltransferase, is the first report on a phage tail-derived pyocin effector targeting a specific regulatory protein (Rho GTPases) in the vertebrate host cell and might also contribute to human disease.62. The results of our study on the effector component Afp18 might serve as a paragon for the fast growing number of phage-like pyocin producing pathogenic bacteria.

Methods

Materials and bacterial strains. DNA modifying enzymes were obtained from Fermentas (St. Leon-Rot, Germany), Thunson High-Fidelity DNA Polymerase from New England Biolabs (Ipswich, MA) and Phusion Ultra HF DNA Polymerase from Stratagene (Waldbrohn, Germany). UDP-[14C]glucose and UDP-[14C]N-acetylglucosamine were from Biotrend (Cologne, Germany). UDP-[14C]galactose was from Perkin-Elmer Life Sciences (Rodgau, Germany).

2′(3′)-O-(N-methylanthraniloyl)-guanosine 5′-diphosphate (mant-GDP) and mant-guanosine 5′-[β,γ-imido]triphosphate (mant-GppNHp) were from Jena Bioscience (Jena, Germany). PET-28a vector was from Novagen (Madison, WI).

pGENX1 and pGEX-4T3 were from GE Healthcare (Freiburg, Germany). Yersinia
ruckeri (ATCC 29473) was from the German Collection of Microorganisms and Cell Cultures (DSM) and cultivated in CASO bouillon at 28 °C. E. coli T7 was used for general cloning and protein expression of pGEX constructs. E. coli BL21 (DE3) (Stratagene) were used for protein expression of PET constructs. Toxin B was prepared from Clastidium difficile supernatants and purified by anion exchange chromatography. The plasmids pGEX-T1-LARG (766–1138) was kindly provided by Mohammad Reza Ahmadian (University Düsseldorf, Germany). All other reagents were of analytical grade and purchased from commercial sources. For all proteins used in this study the accession numbers and the species are listed in Supplementary Table 2.

**Antibodies.** Anti-RhoA (3L74, dilution 1:500), anti-Cdc42 (Cat. No. 17–299, dilution 1:500), -Rac1 (23A8, dilution 1:2500) and anti-GAPDH (6C5, dilution 1:200,000) antibodies were from Millipore (Schwalbach, Germany), anti-GFP (A10261) and anti-deep-stick etch virus (TEV) protein cleavage site-GST-Charge Kit (Stratagene, La Jolla, CA) in combination with PhituPur UF DNA polymerase was used for the replacement of one to three nucleotides using the oligonucleotides shown in Supplementary Table 1. All sequences of corresponding plasmids were confirmed by sequencing (GATC Inc., Konstanz, Germany).

**RhoA purification.** Escherichia coli BL21*CodonPlus cells (Stratagene) transformed with the desired plasmid were grown in LB broth supplemented with 50 μg/ml ampicillin and an introduced tobacco etch virus (TEV) protease cleavage site. Thrombin was removed by binding to benzamidine-Sepharose (GE Healthcare). Bound proteins were eluted with 10 mM reduced glutathione, 0.5 M imidazole, Fast Flow column, according to the manufacturer’s instructions (GE Healthcare).

**Microscopy and imaging acquisition.** For overviews of embryos in 0.3 × 0.176 × 0.176 mm of the corresponding mRNA, followed by lacZ staining (Supplementary Fig. 5b). Apf18p activity in in vitro translated protein was additionally validated by an in vitro glycosylation reaction with recombinant RhoA. For mosaic labelling, plasmid vectors of the following constructs were injected in one blastomere at 16-cell stage: pc2S + vector (Invitrogen, Life Technologies) were used: pcET 28a(+) and pcET 28a(-) were linearized with NotI; pc2S + Apf18p and pc2S + Apf18p Nxn linearized with NotI; pc2S + RHOA and pc2S + RHOA Y34F were linearized with Acc65I. The original construct pGEGF-N1-LifeAct was subcloned into pc2S + vector and linearized with NotI. The pL3- pCS2-HBB-mRFP construct was linearized with NotI. For confocal microscopy, an Axio Observer.Z1 spinning disc (objective: LD C-Apochromat 1.0) and Zen 2012 (blue edition) software was used. Image processing was performed using Adobe Photoshop CS4 Extended. Whole embryo fluorescence images were processed using ImageJ and ZEN software, representing control and experimental samples were adjusted in an identical manner by using the Photoshop ‘levels’ tool to spread the intensity values linearly to fill the 256 values of the RGB spectrum. Images show cells or embryos representative for each experimental condition.

**Cell culture.** HeLa cells (ATCC CCL-2) were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum (FCS), 1% non-essential amino acids, 4 mM penicillin, 4 mM streptomycin and 1% sodium bicarbonate (Biochrom, Berlin, Germany), were cultured in a humidified atmosphere of 5% CO2 at 37 °C. For crystallization, the cells were starved overnight in DMEM without FCS. Danio rerio (ZF4) (ATCC CRL-2050) were cultivated in DMEM/F12 (Biochrom, Berlin, Germany) with 10% FCS, 4 mM penicillin, 4 mM streptomycin and Fungizone (Life Technologies) in 4% CO2 atmosphere at 28 °C. Transfection of cDNA in ZF4. In ZF4. The original construct pGEGP-C1-Afp18p and pGEGP-C1-Afp18p Nxn were processed by using the Metamorph 7.0 software (Universal Imaging, Downingtown, PA).

**Injection of zebrafish embryos.** Danio rerio (Danio rerio) were bred and maintained in our animal facility under standard conditions. For all microinjection methods, WT embryos obtained by single pair mating of 6–18-month-old male and female AB/TL strain zebrafish were used. Injections were performed at the one-cell stage except when indicated otherwise. Holding and breeding of zebrafish were in accord with the German laws for animal care under a permit obtained from the Regierungspärisstum Freiburg. Embryos were incubated in 0.3 × Danieu’s medium or 1 × Danieu’s with 1% v/v Penicillin (5,000 unit/ml) Streptomycin (5,000 μg/ml) solution (Gibco, Life Technologies) at 28.5 °C. Embryos, injected with recombinant proteins mRNA, or vector DNA were staged according to developmental progress of their non-injected littermates. For mRNA synthesis, S6 Message mMachine Kit and T7 Message mMachine Ultra Kit (Ambion, Life Technologies) and the complementary DNAs of the following expression constructs in pc2S + vector (Invitrogen, Life Technologies) were used: pcET 28a(+) and pcET 28a(-) were linearized with NotI; pc2S + Apf18p and pc2S + Apf18p Nxn linearized with NotI; pc2S + RHOA and pc2S + RHOA Y34F were linearized with Acc65I. The original construct pGEGP-N1-LifeAct was subcloned into pc2S + vector and linearized with NotI. For confocal microscopy, an Axio Observer.Z1 spinning disc (objective: LD C-Apochromat × 40/1.1 W) and an Axio Imager Z1 (objective: Plan-Apochromat × 63/1.2 W) for single-plane images was used. Images were acquired with ZEN 2012 software (Zeiss, Jena, Germany).

**Injection of zebrafish embryos.** Embryos were injected with 4% paraformaldehyde in PBS for 2 h at room temperature. After washing with PBST (PBS, 0.5% Triton X-100), the embryos were dechorionated manually. Embryos were dehydrated with methanol (100%) for 0.5 h, 50% for 0.5 h and for 10 min in 100% ethanol. For 1% BSA for 2 h and incubated with primary (anti-GFP and anti-RhoA) antibodies in blocking solution overnight. Embryos were washed 6 h with PBST followed by the secondary antibody (anti-chicken Alexa Fluor 488 and anti-rabbit Alexa Fluor 555) incubation in blocking solution. The embryos were finally washed 5 × in PBST before imaging.

**Immunodetection.** Embryos were fixed with 4% paraformaldehyde in PBS for 2 h at room temperature. After washing with PBST (PBS, 0.5% Triton X-100), the embryos were dechorionated manually. Embryos were dehydrated with methanol (100%) for 0.5 h, 50% for 0.5 h and for 10 min in 100% ethanol. For 1% BSA for 2 h and incubated with primary (anti-GFP and anti-RhoA) antibodies in blocking solution overnight. Embryos were washed 6 h with PBST followed by the secondary antibody (anti-chicken Alexa Fluor 488 and anti-rabbit Alexa Fluor 555) incubation in blocking solution. The embryos were finally washed 5 × in PBST before imaging.
**Glycosylation reaction.** Recombinant AfpG (10 nM if not otherwise stated) was incubated with 10 mM UDP-[3H]-N-acetylglucosamine in a buffer, containing 50 mM Tris-HCl pH 7.4, 10 mM NaCl, 1% NP-40, 10% glycerol, 2 mM MgCl₂, and 0.1 mM phenylmethylsulfonylfluoride; total volume was 20 μL; incubation was performed overnight at 37 °C. After stimulation of recombinant TAG-gentargeted GTP-binding proteins (2 μM) or cell lysate. Total volume was 20 μL; washed proteins were subjected to SDS-PAGE and phosphorimaging. Uncropped gels and autoradiographs from the corresponding figures of the main text are presented in Supplementary Fig. 6. For quantitative modification, GST-RhoA was precipitated by gluthathione-Sepharose beads were modified with Afp1825 (10 nM) and UDP-GlcNAc (100 μM). Beads were extensively washed with glycosylation buffer and RhoA eluted by thrombin cleavage in buffer C (10 mM TEA pH 7.5, 150 mM NaCl and 2.5 mM MgCl₂). The samples were separated on a HPLC-Chip with an analytical column of 75 μm i.d. and 15 cm length. Quantitative glycosylation was proven by a second glycosylation reaction with biotin-aldehyde according to the manufacturer’s instructions (Click-IT Biotin Protein Analysis Detection Kit, Molecular Probes, Darmstadt, Germany) and western blotting using HRP-coupled streptavidin (dilution 1:10,000, Cell Signaling, Danvers, MA).

**Deglycosylation reaction.** Recombinant RhoA (6 μg), TABI (1 μg) or Rho GT-Pases of cell lysate (130 μg) were incubated with 4 μM uridine diphosphate N-acidicglucosamine (UDP-GlcNAc) in the presence of Afp185 (24 nM) or Afp185-Nax (each 24 nM) in a buffer containing 10 mM Hepes, pH 7.5, 2 mM MgCl₂, 1 mM MnCl₂ and 0.1 mg mL⁻¹ BSA for 60 min at 30 °C. Detection of GlcNAc-modified proteins was performed by click chemistry reaction with biotin aldehyde according to the manufacturer’s instructions (Click-IT Biotin Protein Analysis Detection Kit, Molecular Probes, Darmstadt, Germany) and western blotting using HRP-coupled streptavidin (dilution 1:10,000, Cell Signaling, Danvers, MA).

**Mant-GDP- and mant-GppNHp binding.** The measurements were carried out on a LS55B fluorospectrometer from PerkinElmer at 4°C. The amount of nucleotide (GDP/GTP) bound to RhoA was determined by ion-pair reversed-phase liquid chromatography using a C-18 RP column (LiChrosorb 5 μm, 250 × 4 mm) using an Agilent 1100 HPLC system with a calibrated detector system (absorbance of guanine at 252.4 nm). The separation of the nucleotides was performed at ambient temperature with a flow rate of 0.5 mL min⁻¹ with an isocratic phosphates buffer (50 mM, pH 6.5) containing 10 mM tetra-butylammonium bromide and 7.5% acetonitrile. For nucleotide exchange experiments, 98.6 ± 1.3% of RhoA was preloaded with GDP. The nucleotide exchange of RhoA was measured over the time by following the increase in fluorescence intensity at λex = 444 nm (λem = 520 nm) by binding of mant-GDP and mant-GppNHp (each 1 μM) in degassed buffer C (10 mM TEA, pH 7.5, 150 mM NaCl and 2.5 mM MgCl₂). The samples were excited in a cycle of 1 min for 1 s.

**Assay for GAP-stimulated GTPase activity**. RhoA (3.6 μM) was incubated with Afp185 (170 nM) in the absence (RhoA control) or presence UDP-GlcNAc (30 μM). Subsequently, RhoA was loaded with [γ-32P]GTP (20 μM) for 5 min at 37 °C in loading buffer (50 mM Tris-HCl pH 7.5, 10 mM EDTA and 2 mM DTT). MgCl₂ (25 mM, final concentration) and unlabelled GTP (1 mM, final concentration) were added. GAP-stimulated GTPase activity was measured at 25 °C by the addition of the 50 kDa active fragment of p50RhoGAP (550 nM, final concentration). At the indicated time points, proteins were collected by filtration through wet 0.22 μm nitrocellulose filter discs. The filters were washed three times with ice-cold buffer A (50 mM Tris-HCl, pH 7.5, 50 mM NaCl and 5 mM MgCl₂), and protein-bound [γ-32P]GTP was quantified by liquid scintillation counting.

**RhoA-GlcNAc crystallization.** A truncated version of RhoA covering amino acids 1–181 was expressed from a pGEX-based plasmid as GST-fusion protein. The tag was removed by thrombin cleavage in a buffer containing 50 mM Tris-HCl pH 7.5, 100 mM NaCl, 5 mM dithiothreitol and 10% glycerol. After removal of thrombin by benzamidine-sepharose beads, RhoA (15 mg mL⁻¹) was glycosylated by incubation with His-Afp185 (170 nM) in the presence of 1 μM UDP-GlcNAc at 30 °C for 30 min. Quantitative analysis of the glycosylation was performed by a second glycosylation reaction with Afp185 using radiolabelled UDP-[13C]-GlcNAc. After the removal of His-Afp185 by Ni²⁺ affinity chromatography, RhoA-GlcNAc was purified by size-exclusion chromatography Superdex 200 (10/300) in a buffer containing 10 mM HEPES pH 7.4, 50 mM NaCl and 1 mM MgCl₂, concentrated to 7 mg mL⁻¹ and crystallized by sitting drop vapour diffusion method by adding 0.1 M sodium acetate, pH 5 and 1.5 M ammonium sulfate. Crystals were obtained at 20 °C after 6 days with the sitting-drop vapour diffusion method and measured after cryoprotection with 20% glycerol in mother liquid.

**Structure determination.** Diffraction data were collected at 100 K with a Rigaku M007HF X-ray generator and a Saturn 944HG CCD detector. The wavelength was 1.54 Å. Data were processed with HKL3000R. The initial phases were calculated using molecular replacement with MOLREP and a sulfate density envelope with d2m007HF with RhoA (PDB code 1pdf) as a search model using the reflections from 50 to 3.5 Å. The structure was further refined by rigid body and iterative restraint refinement with the software Refmac5 (ref. 68) and model building in COOT69. Structural data are summarized in Table 1. The electron density of the GlcNAc moiety was modeled as alanine. A crystallographic information file with the refinement parameters is deposited with the Protein Data Bank (PDB code 444 CN). Structural images were prepared using PyMOL (http://www.pymol.org/).

**Statistical analysis.** Analysis of variance was performed using GraphPad PRISM. Kruskal–Wallis and Mann–Whitney U-tests were applied. Student’s t-test was applied for pairwise comparison. Fisher’s exact test was applied for statistical analysis of the data set in Fig. 2b, using VassarStats (http://vassarstats.net/).
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Author contributions
T.J. designed the study, performed the experiments, analysed the data and wrote the paper; K.A. designed the study, analysed the data and wrote the paper; S.E. and W.D. performed and analysed the zebrafish injection experiments and wrote the paper; M. Steinemann and C.T. performed experiments, analysed the data; S.W. performed MS analyses; T.J., M. Schimpl, D.M.F.v.A. designed the protein crystallization, performed X-ray analysis and analysed the glyco-RhoA structure; all authors discussed the results and commented on the manuscript.

Additional information
Accession codes: Atomic coordinates and structure factors for the reported crystal structure are deposited at the RCSB data bank under accession code 5A0F.
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