Cell entry of a host-targeting protein of oomycetes requires gp96

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The animal-pathogenic oomycete *Saprolegnia parasitica* causes serious losses in aquaculture by infecting and killing freshwater fish. Like plant-pathogenic oomycetes, *S. parasitica* employs similar infection structures and secretes effector proteins that translocate into host cells to manipulate the host. Here, we show that the host-targeting protein SpHtp3 enters fish cells in a pathogen-independent manner. This uptake process is guided by a gp96-like receptor and can be inhibited by supramolecular tweezers. The C-terminus of SpHtp3 (containing the amino acid sequence YKARK), and not the N-terminal RxLR motif, is responsible for the uptake into host cells. Following translocation, SpHtp3 is released from vesicles into the cytoplasm by another host-targeting protein where it degrades nucleic acids. The effector translocation mechanism described here, is potentially also relevant for other pathogen-host interactions as gp96 is found in both animals and plants.
Oomycetes (or watermolds) are eukaryotic microbes that are among the most devastating pathogens of animals and plants with a huge economic and environmental impact in cultivated as well as natural ecosystems.1-4. Similar to pathogenic fungi, oomycetes can also secret effector proteins that enter the host to establish an infection. They assist the invasion and propagation of the pathogen by reducing the host resistance and overcoming immune responses as well as adapting the host metabolism to the benefit of the pathogen5,6. However, a detailed molecular understanding of the translocation of effector proteins from oomycetes into host cells is missing.

In plant-pathogenic oomycetes from the order Peronosporales, a large group of effector proteins are characterised by an N-terminal RxLR motif (Arg–Xaa–Leu–Arg)7-8. Although, the RxLR motif is highly conserved, its precise role in the translocation mechanism of effectors into host cells is under debate9,10. It is postulated that the RxLR motif of effectors from Phytophthora infestans itself might be involved in the uptake by binding to phospholipids in the host membrane. However, recently it was shown that the RxLR motif of the AVR3a effector from P. infestans is cleaved off before it is secreted from the pathogen11. Following the sequence homology to the PExEL and TExEL motifs in Plasmodium falciparum and Toxoplasma gondii, respectively12,13, to the conserved RxLR motifs in P. infestans and Phytophthora sojae could also work as a sorting signal in the pathogen itself13, which directs the effectors to the export pathway while the translocation into the host is mediated by a translocon16.

Little is known about effector proteins from the fish-pathogenic Saprolegnia parasitica beside the pathogen-independent uptake of SpHtp111. SpHtp1 is expressed during early stages of infection and self-translocates into host cells in a pathogen-independent manner by binding to tyrosin-O-sulphates. Here, we characterise another host-targeting protein (SpHtp3) from S. parasitica and reveal a model for the translocation mechanism. After secretion by S. parasitica, SpHtp3 binds to the surface of the host cell and is taken up via a lipid-raft associated gp96-like receptor. Inside the cell, SpHtp3 is released from its vesicles by another host-targeting protein (SpHtp1). Once in the cytosol, SpHtp3 is able to degrade RNA with its bifunctional nuclease domain. Furthermore, translocation of SpHtp3 into host cells was inhibited with supramolecular tweezers17,18 providing a promising tool for uptake inhibition studies of other host-targeting proteins.

Results
Infection structures of S. parasitica. Little is known about how animal-pathogenic oomycetes infect their hosts because most research has been performed with plant-pathogenic oomycetes that form highly specialised infection structures such as appressoria and haustoria19,20. Like the plant pathogen P. infestans, also the animal pathogen S. parasitica forms an infection structure on the surface of fish cells, which resembles an adhesorium rather than a haustorium (Fig. 1a). The adhesorium remains in place until later stages of infection. Indeed, the pathogen and the host membranes are in close proximity with some contacts and a high number of vesicle-like structures are formed (Fig. 1b) allowing for possible exchange of nutrients and effector proteins as has also been suggested for plant-pathogenic oomycetes and fungi21,22.

Pathogen-independent translocation of SpHtp3 into host cells. Although effector proteins are essential to establish an infection, their pathogen-independent translocation and the exact translocation route into the host are not clear23-12. To investigate the translocation process of host-targeting proteins secreted by S. parasitica, we have used SpHtp3 (S. parasitica host-targeting protein 3) as a model protein since it contains characteristics typical for effector proteins. SpHtp3 comprises a signal peptide for secretion, an RxLR sequence (Arg–Thr–Leu–Arg) and the effector domain is a putative Staphylococcal nuclease domain (SNase, E value: 7.3e−23, Pfam-A ID: PF00565) (Fig. 2a). In addition, SpHtp3-like genes can be found in more than 40 other species being pathogenic to animal and plants (Supplementary Table 1). As expected by the conserved active site, recombinant SpHtp3 shows RNA as well as DNA degradation activity (Fig. 2b) like the Staphylococcus aureus nuclease23. The specific activity of SpHtp3 was determined by real-time fluorescence imaging to be 30 nmol min⁻¹ mg⁻¹ (kcat: 0.024 s⁻¹), which is also similar to the activity of SNase (Fig. 2c) and shows a general salt dependency with a clear reduction by Mg²⁺ and SO₄²⁻ ions (EC₅₀ = 0.35 mM for MgSO₄, Supplementary Fig. 1a and b). RNA degradation by a possible RNase contamination from the
purification process could be excluded by control experiments (Supplementary Fig. 1c–e).

In addition, we have used a recombinant protein construct of SpHtp3 fused to mRFP to investigate the translocation ability into living RTG-2 trout fibroblast cells (Fig. 2d and Supplementary Fig. 1f). Indeed, SpHtp3 showed self-translocation into fish cells and is located in vesicle-like structures. However, in contrast to SpHtp1, the uptake of SpHtp3-mRFP into RTG-2 cells is more efficient at a lower pH of 5.5. The pH-dependency of the SpHtp3 uptake could also be observed in other fish cell lines (Supplementary Fig. 1g) but is unlikely to be caused by structural changes considering the highly overlapping CD spectra of SpHtp3 at pH 5.0, 6.0 and 7.0 (Supplementary Fig. 1h).

SpHtp3 is taken up into host cells via its C-terminus. We have chosen SpHtp3 as a model protein since it also contains an RxLR motif, which is thought to be involved in the translocation of effectors from plant-pathogenic oomycetes6. However, the translocation of SpHtp3 appears to be RxLR-independent since a C-terminal truncated version of SpHtp3 (21–55 aa), containing the RxLR motif, does not translocate into RTG-2 cells (Fig. 3a). Whereas, an RxLR mutant (RTLRT/LGLG) of the full-length RTG-2 cell RNA) and DNA (right, linearised pET21b) degrading activities of SpHtp3-His6 and SpHtp3-mRFP (n = 3). c Real-time ribonuclease activity assessment of SpHtp3 wt (black) compared to a negative control (SpHtp1-mRFP, red) and a non-functional mutant of SpHtp3 (GTLG, blue) with RNaseAlert® (n = 2). d Autonomous translocation activity of recombinant SpHtp3-mRFP into living RTG-2 cells at pH 7.5 and 5.5. The control (mRFP only) does not show any translocation. Scale bar: 20 µm (n = 3)

SpHtp3 is a self-translocating nuclease. a Amino acid sequence of SpHtp3 (top), including the secretion signal (M1-G21, underlined), the RxLR sequence (R48-R51, red) and the predicted nuclease domain (L89-S197, bold). Protein domain structure of SpHtp3 (bottom). b Visualisation of RNA (left, RTG-2 cell RNA) and DNA (right, linearised pET21b) degrading activities of SpHtp3-His6, and SpHtp3-mRFP (n = 3). c Real-time ribonuclease activity assay of SpHtp3 wt (black) compared to a negative control (SpHtp1-mRFP, red) and a non-functional mutant of SpHtp3 (GTLG, blue) with RNaseAlert® (n = 2). d Autonomous translocation activity of recombinant SpHtp3-mRFP into living RTG-2 cells at pH 7.5 and 5.5. The control (mRFP only) does not show any translocation. Scale bar: 20 µm (n = 3)

A gp96-like protein is a host receptor of SpHtp3. To narrow down the cell entry mechanism of SpHtp3, fish cells were treated with different compounds known to inhibit key factors of common cell entry pathways (Fig. 4a). The inhibitor studies support a lipid raft dependent-translocation process for SpHtp3 (nystatin) and excluded clathrin-mediated endocytosis (dynasore). The entry of SpHtp3 into host cells is increased with increasing temperatures, supporting a lipid-mediated process dependent on the fluidity of the cell membrane (Supplementary Fig. 3a). On the other hand, the uptake of SpHtp3 into RTG-2 cells is saturable (Fig. 4b), which excludes a purely diffusion-driven process. In contrast to SpHtp3, another effector protein from S. parasitica SpHtp1, expressed during earlier stages of infection14, is translocated via a different route (inhibitor of clathrin-mediated endocytosis, Supplementary Fig. 3b). To identify a potential receptor for SpHtp3, we have performed 2D-PAGE analysis of proteins from trout RTG-2 cells incubated with and without SpHtp3 that revealed an additional spot corresponding to a gp96-like protein with 78% identity (GPR94, endoplasmin; GSONMT00046981001) (Fig. 4c). In the next step, we performed a pull down experiment of an RTG-2 cell lysate with SpHtp3 to proof a complex formation (Fig. 4d). An additional band appeared at the size of gp96 and LC-MSMS analysis confirmed the presence of peptides of a gp96-like protein (GSONMT00046981001). The direct interaction between recombinant SpHtp3-His6 and recombinant gp96 (no tag) was also confirmed by a cross-link experiment, which revealed another band at the height of a complex (130 kDa) detected by an α-His antibody (Fig. 4e). To investigate the potential role of the gp96-like protein in the translocation of SpHtp3, we have knocked down gp96 in human A549 cells (Fig. 4f) resulting in a clear reduction in intracellular localised SpHtp3-mRFP vesicles (Fig. 4g). Interestingly, and in agreement with the pH-dependency of the uptake of SpHtp3 into fish RTG-2 cells and
human A549 lung cells (Fig. 2d and Supplementary Fig. 3c), is the relocalisation of gp96 from the cell centre to the cell membrane in human cells at a lower pH (Fig. 4h). Furthermore, the uptake of SpHtp3 into A549 cells was reduced remarkably by the simultaneous co-incubation with an α-gp96 antibody compared to a control with SpHtp3 only (24 vs. 2 vesicles per cell on average) (Supplementary Fig. 3d).

A supramolecular compound inhibits translocation of SpHtp3. The C-terminus of SpHtp3 is rich in Lys and Arg, and thereby a promising target for supramolecular hydrogen phosphate tweezers. These tweezers are supramolecular ligands designed to modulate protein–protein interactions by specifically targeting defined areas on a protein in a predictable manner. Hydrogen phosphate tweezers bind selectively Lys and Arg on protein surfaces by drawing the positively charged side chains of Lys and Arg into the tweezers’ tailored cavity. To investigate the interaction between the tweezers and the SpHtp3 translocation module, we have calculated the solution structure of the C-terminus (Fig. 5a). In line with the homology model (Fig. 3c), the peptide forms a short but highly positively charged helix (Fig. 5b). The peptide with the double mutant (K208A/R210A) retains the helical character but with a reduced positive charge due to the replacement of the positively charged Lys/Arg by the hydrophobic Ala (Fig. 5c). We used the structure information for H-1D titration experiments of the peptide with a stepwise increasing amount of tweezers (1:200–1:4 tweezers:SpHtp3) (Fig. 5d). Since Arg11 and Lys12 show already a loss of signal intensities at low SpHtp3:tweezers ratios (200:1), these residues are expected to be the main binding sites and indicate a high affinity binding constant ($K_D$ in the lower μM range). Other signals (Lys9 or Ala10 that are also a part of the motif) undergo only weak changes pointing towards a close proximity to the tweezers, while signals for the remaining residues underwent only unspecific changes and are not involved in the peptide–tweezers complex formation.

Next, we tested the potential of the tweezers to block the translocation of SpHtp3 into RTG-2 cells (Fig. 5e). At lower concentrations of the tweezers, only the uptake of SpHtp3 into cells is blocked, which leads to an accumulation at the cell surface. However, with higher tweezers concentrations binding of SpHtp3 to the cell membrane is also interrupted indicating a two-step translocation process of SpHtp3 as was already observed in the α-gp96 blocking experiment (Supplementary Fig. 3d).

As a proof of concept, we have tested if molecular tweezers were able to prevent an infection of Galleria mellonella larvae by S. parasitica. For this, protoplasts were either pre-incubated with molecular tweezers for 1 h or co-injected with molecular tweezers into larvae (Fig. 6a, b). While larvae injected with protoplasts only died 3 dpi, pre-incubated protoplasts did not cause any mortality and although statistically not significant, protoplast that were co-injected with molecular tweezers showed a delay in mortality of larvae.
Vesicle release of SpHtp3 is mediated by another effector. During our infection studies of RTG-2 cells by *S. parasitica*, we could observe the degradation of cytoplasmic RNA visualised by SytoRNA of cells that are in direct contact with hyphae of *S. parasitica*, while the nuclei of infected cells remain intact (Fig. 7a). Hence, we conclude that SpHtp3 or similar, unidentiﬁed nucleases must have been translocated into the host cytosol. However, SpHtp3 is the only protein comprising a secretion signal, a predicted bi-functional nuclease domain, without a nuclear localisation signal (which is in line with the cytoplasmic localisation) and up-regulated during infection. To investigate the effect of SpHtp3 under infectious conditions, we have pre-incubated RTG-2 cells with recombinant SpHtp3-mRFP. After SpHtp3 was taken up into vesicles, pre-treated cells were co-incubated with *S. parasitica*. Indeed, in fish cells that are in direct contact with hyphae of *S. parasitica*, vesicles ﬁlled with recombinant SpHtp3-mRFP disappeared (Fig. 7b and Supplementary Movie 1). Remarkably, the number of ﬂuorescent SpHtp3 vesicles was only reduced in cells with direct hyphal contact compared to non-infected cells (75% and 17%, **p < 0.001, ***p < 0.0001 (t-test). Scale bar: 20 µm. Graph represents the ﬂuorescence intensity of one cell for each pH as indicated by dashed lines (n = 2).**
respectively) (Fig. 7c), which indicates a cofactor-mediated release of SpHtp3.

Previously we found that SpHtp1 from S. parasitica is expressed during early stages of infection and also able to enter host cells. SpHtp1 is an intrinsically disordered protein with a high degree of flexibility, which is characteristic for the mediation of protein–protein interactions. To investigate a potential interaction between SpHtp1 and SpHtp3, we pre-incubated both proteins...
proteins before performing the translocation assay into RTG-2 cells (Fig. 7d). At pH 7.5, the translocation of SpHtp3 into vesicles is reduced, which is reflected by the low amount of vesicles with a low fluorescence intensity (Fig. 7e). After pre-incubating with SpHtp1 vesicles, the periphery of the cell disappear and the cytosolic RFP fluorescence is increased. This might indicate a potential role of SpHtp1 in the uptake of SpHtp3 at a neutral pH (pH 7.5) and its release from vesicles. Indeed, co-incubation of recombinant SpHtp1 and SpHtp3 (mRFP- or His-tagged) in vitro, resulted in an additional band for a cross-linked SpHtp1–SpHtp3 complex, which was confirmed by LC-MSMS analysis (Fig. 7f, Supplementary Table 2).

**Fig. 7** SpHtp3 is released from vesicles with the help of SpHtp1 from *S. parasitica*. **a** RTG-2 cells in direct contact with *S. parasitica* are shrunk with a condensed nucleus. In these cells, no cytosolic RNA (SytoRNA) can be detected and infected cells contain a high amount of vesicles (membrane stain FM4-64FX, see also Fig. 1b). In contrast, cells in close proximity but no direct contact do not show any morphological abnormalities (*). Scale bar: 20 µm (*n = 3*). **b** RTG-2 cells (c) were challenged with *S. parasitica* (h) after 1 h incubation with SpHtp3-mRFP. A hyphal tip (arrowhead, DIC) is attacking an RTG-2 cell. Magnification of the infected cell (red square) at different time points (bottom) show vesicles disappearing within a minute (arrowheads). See also Supplementary Movie 1. In contrast, cells in close proximity but no direct contact to *S. parasitica* contain less disappearing vesicles (*). Scale bar: 20 µm (*n = 3*). **c** Quantification of SpHtp3-mRFP containing vesicles of RTG-2 cells from **b** over time. **d** Vesicle release of SpHtp3-mRFP into the cytosol of RTG-2 cells after pre-incubation with SpHtp121–198-His6 at pH 7.5. SpHtp3 accumulates in vesicles of RTG-2 cells after self-translocation (see also Fig. 2d). However, after co-incubation of SpHtp1 with SpHtp3, the number of vesicles in the periphery of the cells is reduced and the cytosolic fluorescence of RFP increased. Pictures were taken with a Zeiss Imager M2. Scale bar: 20 µm (*n = 2*). **e** Fluorescence intensity of SpHtp3-mRFP across the cell as indicated by dashed lines in **d**. **f** In vitro complex formation of recombinant SpHtp1-His6 and SpHtp3-His6 after cross-link verified by LC-MS/MS (Supplementary Table 2). An additional band, which only appears in the sample with both proteins is highlighted (Complex).
Discussion

For the successful establishment of an infection, plant-pathogenic oomycetes use a broad spectrum of secreted effector proteins to promote the infection process by mediating invasion and suppressing host immune responses. Currently, the pathogen-independent self-translocation of effector proteins secreted by plant-pathogenic oomycetes is unclear. The present study with SpHtp3 from the fish-pathogenic oomycete *S. parasitica* reveals a potential model for effector translocation from oomycete into host cells. Interestingly, uptake studies with the SpHtp3 homologue from the plant-pathogenic oomycete *P. sojae* (PsHtp3) into fish cells confirmed the pathogen-independent self-translocation also in non-host cells (Supplementary Fig. 4a). Homologues of the gp96 receptor are also present in several plant species with an identity of 51%, but a potential role for the uptake of effector proteins into plant cells remains to be investigated.

The largest group of effector proteins of the well-known plant-pathogenic oomycetes, *P. sojae* and *P. infestans*, is characterised by a highly conserved RxLR motif (Arg–Xaa–Leu–Arg), which is thought to play a role during the secretion and/or translocation of effectors into plant cells. However, the RxLR motif of SpHtp3 from *S. parasitica* is not involved in the translocation process. Instead, the C-terminus is involved in the negatively charged cell membrane of the host, like the positively charged lysine patches on the effector domains of AVR3a from *P. infestans* and AVR1b from *P. sojae*. Regarding the uptake process, the attachment and invasion of *Escherichia coli* to human endothelial cells (HBMEC) are two independent and different processes mediated by the same motif similar to our observation with SpHtp3.

After binding to the cell, SpHtp3 is taken up into fish cells mediated by a gp96-like protein located in lipid rafts, which is a very ancient evolutionary way of translocating molecules. The gp96-mediated translocation has a low affinity but a high capacity, which is perfectly suited for the uptake of a huge reservoir of effectors secreted by pathogens. This is supported by a large number of vesicles, which can be observed in single cells infected with *S. parasitica* (Figs. 1b and 6b). Normally, gp96 is a chaperone of the Hsp90 family located in the ER but is also found exposed to the cell surface. The environmental acidification by *S. parasitica* (Supplementary Fig. 4b) and the reduction of the extracellular space during an infection result in an increased exposure of gp96 to the cell surface (Fig. 4h). The ubiquitous expression of gp96 in several tissues as well as across a broad range of species results in a potential receptor function for several pathogens ranging from bacteria like *E. coli* to fungi like *Candida albicans* and the vesicular stomatitis virus.

gp96 occurs naturally as a mixture of non-glycosylated and hyper-glycosylated molecules with up to five glycosylation sites inserted probably by a sequential mechanism. It is postulated that the deglycosylated form has a higher affinity to substrates. Thus, we speculate that the lower spot of gp96 after SpHtp3 incubation on 2D-PAGE (Fig. 4c) is the deglycosylated moiety of gp96, which has bound SpHtp3 and was relocalised into the cell. Another posttranslational modification of gp96 is the phosphorylation of tyrosine residues by the Fyn kinase in the ER, which is important for the shuttling of gp96 between the ER and the cell membrane. Consequently, the inhibition of tyrosine kinases by genistein leads to the intracellular trapping of gp96 in line with our studies, the SpHtp3 translocation is clearly reduced (Supplementary Fig. 4c). During the re-uptake of gp96 with or without a ligand, gp96 interacts with sulfated sites of lectins (OS-9) and heparin/HSPG. Hence, after treating cells with the sulfation inhibitor NaClO, the binding of gp96 to the cell surface is abolished in the same way as the uptake of SpHtp3 into fish cells (Supplementary Fig. 4d). Interestingly, SpHtp1 also translocates into host cells in an O-sulfation dependent manner. However, both effector proteins are expressed during different stages of infection and are clearly using different pathways into the host cell. Therefore, we speculate that both effectors attach to the host membrane by ionic interactions, but are using different receptor molecules to enter the host cell.

Our research has given novel insights into how a fish-pathogenic oomycete establishes an infection. The single most limiting factor for sustainable expansion of fish farming is disease. Indeed, *S. parasitica* is responsible for major losses in the aquaculture industry. Therefore, a detailed understanding of the
infection processes at the molecular level is very important for the development of new control strategies against oomycetes that will address global challenges in sustainable food security.

**Methods**

**Cloning of SpHtp3 constructs.** SpHtp321−211 lacking the putative N-terminal signal peptide was amplified from a cDNA of *S. parasitica* using the KOD Hot start polymerase (Novagen) according to the manufacturer’s protocol. Primer: fw (+ Ndel) 5′-TACAGCTATGCTGCTAGCCGGTCTCG-3′ and rv (+ EcoR I) 5′-ATCGATGATCTTTGATCCGGGGCGGTCTG-3′ [58]. PCR fragment was cloned into the pGEM-T easy vector (Promega) according to manufacturer’s instruction in DMEM (Gibco) supplemented with 10% FBS, 100 units ml−1 Penicillin and 100 μg ml−1 Streptomycin. Cells were plated onto glass coverslips and incubated to a confluence of 90–95%. Cells were washed with PBS, incubated with PFA (2% v/v in PBS, 4 °C) and the pellet re-suspended in 40 ml 25 mM NaPi buffer (pH 7.5, 4 °C) and pellets re-suspended in 40 ml 25 mM NaPi buffer (pH 7.5) supplemented with 30 mM imidazole. SpHtp321−211 fragment was then cloned into pET21b-mRFP using the Ndel/EcoRI cut sites. The RTRL/GTLG mutant was purchased from GenScript and sub-cloned into pET21b-mRFP. The K208A/R210A double mutant was prepared with the Q5 Site-Directed Mutagenesis Kit (#E0554, NEB). All constructs were verified by sequencing (Source Bioscience).

**Expression and purification of recombinant proteins.** Purification procedure is exemplarily shown for SpHtp3-mRFP. Various SpHtp3 constructs were transformed into E. coli Rosetta gami B (DE3, pLysS, #71137, Novagen) cells. Cells were inoculated and grown in LB media to an OD600 of 0.8 and protein expression was subsequently induced with 1 mM IPTG for 6 h at 37 °C. Cultures were centrifuged (10 min, 15,000×g, 4 °C) and pellets re-suspended in 20 ml 50 mM NaPi buffer (pH 7.5) supplemented with 250 μM benzamide (#E1014, Sigma), two tablets of protease inhibitor containing 1693519001 and 1 ml complete. Cell lysis was performed with 0.1 g lysozyme (#62971, Fluka) for 20 min at 4 °C and successive French-Press. Cell debris and aggregates were pelleted by centrifugation (1 h, 48,000×g, 4 °C) and the supernatant applied to a QAE agarose column equilibrated with 25 mM NaPi, (pH 7.5). The flow-through was subsequently loaded onto an Ni-NTA agarose column, followed by one wash step with 25 mM NaPi, (pH 7.5) and another one with 25 mM NaPi, (pH 7.5) supplemented with 30 mM imidazole. SpHtp3-mRFP was eluted with 300 mM imidazole. The elution fraction was then applied to a S20, column. Two washing steps followed: 25 mM NaPi, (pH 7.5) and 25 mM NaPi, (pH 7.5) with 50 mM NaCl. SpHtp3 was eluted with 25 mM NaPi, (pH 7.5) containing 500 mM sodium chloride and 10 mM magnesium sulphate for protein stabilisation. Fractions containing SpHtp3-mRFP were pooled and stored at −20 °C.

**Electron microscopy (SEM and TEM).** For TEM, the dehydration is followed by three incubations in acetone for 1 h. Samples were then incubated in increasing concentrations of ethanol (30, 70, 90 and 100% (v/v); 30 min each). For TEM, the dehydration is followed by three incubations in acetone for 1 h. Samples were then incubated in increasing concentrations of ethanol (30, 70, 90 and 100% (v/v); 30 min each). For TEM, the dehydration is followed by three incubations in acetone for 1 h. Samples were then incubated in increasing concentrations of ethanol (30, 70, 90 and 100% (v/v); 30 min each). For TEM, the dehydration is followed by three incubations in acetone for 1 h. Samples were then incubated in increasing concentrations of ethanol (30, 70, 90 and 100% (v/v); 30 min each). For TEM, the dehydration is followed by three incubations in acetone for 1 h. Samples were then incubated in increasing concentrations of ethanol (30, 70, 90 and 100% (v/v); 30 min each). For TEM, the dehydration is followed by three incubations in acetone for 1 h. Samples were then incubated in increasing concentrations of ethanol (30, 70, 90 and 100% (v/v); 30 min each). For TEM, the dehydration is followed by three incubations in acetone for 1 h. Samples were then incubated in increasing concentrations of ethanol (30, 70, 90 and 100% (v/v); 30 min each). For TEM, the dehydration is followed by three incubations in acetone for 1 h. Samples were then incubated in increasing concentrations of ethanol (30, 70, 90 and 100% (v/v); 30 min each). For TEM, the dehydration is followed by three incubations in acetone for 1 h. Samples were then incubated in increasing concentrations of ethanol (30, 70, 90 and 100% (v/v); 30 min each). For TEM, the dehydration is followed by three incubations in acetone for 1 h. Samples were then incubated in increasing concentrations of ethanol (30, 70, 90 and 100% (v/v); 30 min each). For TEM, the dehydration is followed by three incubations in acetone for 1 h. Samples were then incubated in increasing concentrations of ethanol (30, 70, 90 and 100% (v/v); 30 min each). For TEM, the dehydration is followed by three incubations in acetone for 1 h. Samples were then incubated in increasing concentrations of ethanol (30, 70, 90 and 100% (v/v); 30 min each). For TEM, the dehydration is followed by three incubations in acetone for 1 h. Samples were then incubated in increasing concentrations of ethanol (30, 70, 90 and 100% (v/v); 30 min each). For TEM, the dehydration is followed by three incubations in acetone for 1 h. Samples were then incubated in increasing concentrations of ethanol (30, 70, 90 and 100% (v/v); 30 min each).
indicated (Thermo Scientific). Proteins were removed by heating the samples at 65 °C for 5 min. Samples were analysed on a 1% agarose gel (originals are included in the Supplementary Information).

Kinetic measurements of ribonuclease activity. Real-time fluorescent monitoring of ribonuclease activity was performed at RT using the RNaseAlert(R) Lab (Thermo Fisher) in L-15 medium. The reaction was started with 25 pmol of a fluorescent substrate (excitation: 490 nm; emission: 520 nm) and subsequently monitored by a PL9200 fluorescence spectrometer (Edinburgh Instruments) using a 4 × 4 mm² stirred cuvette.

In vitro infection assays with S. parasitica. Culture conditions and zoospore/cyst production of S. parasitica (CBS223.65, originally isolated from young pike (Esox lucius)) was described previously1. RTG-2 cells were grown to 70% confluence in liquid cultures on glass coverslips. For the infection of RTG-2 cells with S. parasitica, 3750 zoospores/cysts were diluted in HBBS supplemented with 10% FBS and 30% L-15 medium. Zoospores/cysts were added to the cells and incubated for 14 h at 24 °C.

For bright field microscopy, infected cells were visualised with an Evox XL light microscope. For fluorescence microscopy, cells were washed after co-incubation of cells and spores 3× with HBBS and fixed with 4% ice-cold PFA in PBST (PBS−0.1% tween) for 15 min at RT. Residual PFA was removed with 3× washing steps with PBS and RTG-2 cells/S. parasitica hyphae were stained with SytoRNA (1:10,000, #S32703, Life Technologies) for 20 min at RT in the dark. Remaining dye was removed with 3× washing steps with PBS. Successively, membrane was stained by FM4-64FX (2 nM, #F34653, Life Technologies) for 5 min on ice in the dark. Remaining dye was removed with 3× washing steps with PBS. Cells were mounted with Vectashield with DAPI. Samples were analysed by a Zeiss LSM710 confocal microscope.

Vesicular release of SpHtp3: RTG-2 cells were grown to confluence and incubated with 3 μM SpHtp321-211-mRFP for 1 h at 18 °C. Cells were washed 3× with L-15 medium andonce with HBBS (Gibco) to remove non-translocated protein as well as remaining nutrients. Subsequently, 1 ml of zoospores/cysts (~3750 cells ml−1) of S. parasitica in HBBS supplemented with 3% FBS and 30% L-15 medium were added to the cells. Cells were co-incubated with the zoospores/cysts for another 3 h at 18 °C and SpHtp3-mRFP was monitored by confocal microscopy with a Zeiss LSM 510 confocal microscope equipped with a water dipping lens. Translation and release from vesicles of SpHtp321-211-mRFP was investigated for 70 min at RT. In total 70 frames were taken, each with a stack of 10 optical slices (z-series) to detect also moving vesicles. Shown are the Z-projections for the time step as indicated which were also used to analyse the decreasing mRFP fluorescence over time with ImageJ. Number of particles was counted for the infected cell compared to all non-infected cells.

In vivo infection assay with Galleria mellonella. An infection assay with G. mellonella was used to determine the effect of the molecular tweezers on the virulence of S. parasitica. Five larvae per group were injected with corresponding mutant was dissolved in 600 μl (90% H2O, 10% D2O) 50 mM NaP buffer (pH 7.3). Standard 2D spectra (NOEY, TOCSY, COSY) were used for backbone assignment. For titration experiments H1-1D spectra were recorded with 128 scans. Data were processed with Topspin 3.0 (Bruker).

NMR spectroscopy and structure calculation. NMR experiments were performed on a 700 MHz Ultrashield NMR spectrometer (Bruker, Ettlingen, Germany) equipped with a cryoprobe (Bruker Biospin). 2 mM of the C-terminal peptide of SpHtp3 (KRETPAQYKARKMNSSVD, FITC-coupled) or the corresponding mutant was dissolved in 600 μl (90% H2O, 10% D2O) 50 mM NaP buffer (pH 7.3). Standard 2D spectra (NOEY, TOCSY, COSY) were used for backbone assignment. For titration experiments H1-1D spectra were recorded with 128 scans. Data were processed with Topspin 3.0 (Bruker).

Homology modelling. Homology models were generated using the YASARA Structure suite with parameters given in Table 1. Molecular dynamics simulations were performed applying a YAMBER2 force field with the YASARA Structure suite using default parameters and 1 ns time steps. Electrostatic potentials were calculated using the Adaptive Poisson-Boltzmann Solver (APBS) applying the YAMBER2 force field.

Statistics. Live cell imaging was repeated at least three times for each condition and quantitative analysis was performed by FACs analysis. Experiments with fixed cells were performed at least three times (Fig. 3f twice) with the same result. Quantitative analysis is shown for one experiment of 50 cells from at least 10 different visual fields exemplarily. Comparison of two groups was done with t-test and for more than two groups, one-way ANOVA was applied. The real-time nucleic activity was measured twice (Fig. 2c) and the live cell imaging infection assay was performed twice (Fig. 7b). Error bars indicate s.e.m. A log rank test was performed for the survival curves of the in vivo infection experiment (co-injection/preincubation vs. control). The results show an increase in survival rate of co-injected tweezers, but this was not statistically significant (p > 0.1).
Data availability. The authors declare that data supporting the findings of this study are available within the paper and its supplementary information files. Assignment and calculation data that support the findings of the structure of the C-terminal peptide of SpHtp3 are available from the corresponding author upon request.

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References
1. van West, P. Saprolegnia parasitica, an oomycete pathogen with a fishy appetite. New challenges for an old problem. Mycologist 20, 99–104 (2006).
2. Phillips, A. J., Anderson, V. L., Robertson, E. J., Secombe, C. J. & van West, P. New insights into animal pathogenic oomycetes. Trends Microbiol. 16, 13–19 (2008).
3. Schornack, S. et al. Ten things to know about oomycete effectors. Mol. Plant Pathol. 10, 795–803 (2009).
4. Derevnina, L. et al. Oomycete RXLR effectors. Functional and structural redundancy and durable disease resistance. Curr. Opin. Plant Biol. 11, 373–379 (2008).
5. Birch, P. R. J. et al. Oomycete RXLR effectors. Functional and structural redundancy and durable disease resistance. Curr. Opin. Plant Biol. 11, 373–379 (2008).
6. Birch, P. R. J., Rehmany, A. P., Pritchard, L., Kamoun, S. & Beynon, J. L. Trafficking arms. Oomycete effectors enter host plant cells. Trends Microbiol. 14, 8–11 (2006).
7. Whisson, S. C. et al. A translocation signal for delivery of oomycete effector proteins into host plant cells. Nature 450, 115–118 (2007).
8. Kale, S. D. et al. External lipid PIP3 mediates entry of eukaryotic pathogen effectors into plant and animal host cells. Cell 142, 284–295 (2010).
9. Ellis, J. G. & Dodds, P. N. Showdown at the RXLR motif. Serious differences of
10. Yaeno, T. et al. Phosphatidylinositol monophosphate-binding interface in the
11. Wawra, S. et al. The RxLR motif of the host targeting effector AVR3a of
12. Sun, F. et al. Structural basis for interactions of the
13. Wawra, S. et al. Fundamental roles of the Golgi-associated
14. Hammoudi, P.-M. et al. Fundamental roles of the Golgi-associated
15. Fokkens, M., Schrader, T. & Klärner, F.-G. A prototypic study in contemporary enzymology. I isolation; physical and
16. Argon, Y. & Prasadarao, N. V. A prototypic study in contemporary enzymology. I isolation; physical and
17. Tucker, P. W., Hazen, E. E. & Cottom, F. A. Staphylococcal nuclease reviewed. A prototypic study in contemporary enzymology. I isolation; physical and
18. Tucker, P. W., Hazen, E. E. & Cottom, F. A. Staphylococcal nuclease reviewed. A prototypic study in contemporary enzymology. I isolation; physical and
19. Grenville-Briggs, L. J. & van West, P. The biotrophic stages of oomycete
20. Tucker, P. W., Hazen, E. E. & Cottom, F. A. A prototypic study in contemporary enzymology. I isolation; physical and
21. Tucker, P. W., Hazen, E. E. & Cottom, F. A. A prototypic study in contemporary enzymology. I isolation; physical and
22. Tucker, P. W., Hazen, E. E. & Cottom, F. A. A prototypic study in contemporary enzymology. I isolation; physical and
23. Tucker, P. W., Hazen, E. E. & Cottom, F. A. A prototypic study in contemporary enzymology. I isolation; physical and
24. Tucker, P. W., Hazen, E. E. & Cottom, F. A. Staphylococcal nuclease reviewed. A prototypic study in contemporary enzymology. I isolation; physical and
25. Tucker, P. W., Hazen, E. E. & Cottom, F. A. Staphylococcal nuclease reviewed. A prototypic study in contemporary enzymology. I isolation; physical and
26. Tucker, P. W., Hazen, E. E. & Cottom, F. A. Staphylococcal nuclease reviewed. A prototypic study in contemporary enzymology. I isolation; physical and
27. Tucker, P. W., Hazen, E. E. & Cottom, F. A. Staphylococcal nuclease reviewed. A prototypic study in contemporary enzymology. I isolation; physical and
28. Tucker, P. W., Hazen, E. E. & Cottom, F. A. Staphylococcal nuclease reviewed. A prototypic study in contemporary enzymology. I isolation; physical and
29. Tucker, P. W., Hazen, E. E. & Cottom, F. A. Staphylococcal nuclease reviewed. A prototypic study in contemporary enzymology. I isolation; physical and
containing the sequence Gal alpha 1-3Gal beta 1-4GlcNAc. Infect. Immun. 53, 573–581 (1986).

51. Na, X., Kim, H., Moyer, M. P., Pothoulakis, C. & LaMont, J. T. gp96 is a human colonocyte plasma membrane binding protein for Clostridium difficile toxin A. Infect. Immun. 76, 2862–2871 (2008).

52. Liu, Y., Mittal, R., Solis, N. V., Prasadara, N. V. & Filler, S. G. Mechanisms of Candida albicans trafficking to the brain. PloS Pathog. 7, e1002305 (2011).

53. Cala, S. E. GRP94 hyperglycosylation and phosphorylation in S21 cells. Biochim. Biophys. Acta 1496, 296–310 (2000).

54. Suriano, R. et al. Differences in glycosylation patterns of heat shock protein, gp96. Implications for prostate cancer prevention. Cancer Res. 65, 6466–6475 (2005).

55. Pagetta, A. et al. Structural insights into complexes of glucose-regulated Protein94 (Grp94) with human immunoglobulin G. relevance for Grp94-IgG complexes that form in vivo in pathological conditions. PloS One 9, e86198 (2014).

56. Asquith, K. L., Baleato, R. M., McLaughlin, E. A., Nixon, B. & Aitken, R. J. Tyrosine phosphorylation activates surface chaperones facilitating sperm-zona recognition. J. Cell Sci. 117, 3645–3657 (2004).

57. Frassetto, A. et al. Grp94 is Tyr-phosphorylated by Fyn in the lumen of the endoplasmic reticulum and translocates to Golgi in differentiating myoblasts. Biochim. Biophys. Acta 1793, 239–252 (2009).

58. Seidler, P. M. et al. Characterization of the Grp94/OS-9 chaperone–lectin complex. J. Mol. Biol. 426, 3590–3605 (2014).

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Author contributions

F.T., L.L., S.W., T.S., P.B. and P.v.W. designed the study. F.T., L.L., S.W., E.D., An.W., N. A.I., I.d.B., K.M., Ar.W., A.T. and T.R. performed experiments. F.T., L.L., S.W., J.D.-U., T.S., P.B., C.J.S. and P.v.W. helped in writing the manuscript. Funding was provided by T.S., P.B., C.J.S. and P.v.W.

Additional information

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Competing interests: F.T. and P.v.W. are listed as inventors in a related patent application, entitled “Particle translocation into Eukaryotic cells” (RKA/BP7352784). The remaining authors declare no competing interests.

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