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The chlamydial OTU domain-containing protein *Chla*OTU is an early type III secretion effector targeting ubiquitin and NDP52

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Summary

Chlamydia **are obligate intracellular pathogens. Upon contact with the host, they use type III secretion to deliver proteins into the cell, thereby triggering actin-dependent entry and establishing the infection. We observed that** ***Chlamydia caviae*** **elicited a local and transient accumulation of ubiquitinated proteins at the entry sites, which disappeared within 20 min. We investigated the mechanism for the rapid clearance of ubiquitin. We showed that the OTU-like domain containing protein CCA00261, predicted to have deubiquitinase activity, was detected in infectious particles and was a type III secretion effector. This protein is present in several** ***Chlamydia*** **strains, including the human pathogen** ***Chlamydia pneumoniae*****, and we further designate it as** ***Chla*****OTU. We demonstrated that** ***Chla*****OTU bound ubiquitin and NDP52, and we mapped these interactions to distinct domains. NDP52 was recruited to** ***Chlamydia*** **entry sites and was dispensable for infection and for bacterial growth.** ***Chla*****OTU functioned as a deubiquitinase** ***in vitro*****. Heterologous expression of** ***Chla*****OTU reduced ubiquitin accumulation at the entry sites, while a catalytic mutant of the deubiquitinase activity had the opposite effect. Altogether, we have identified a novel secreted protein of chlamydiae.** ***Chla*****OTU targets both ubiquitin and NDP52 and likely participates in the clearance of ubiquitin at the invasion sites.**

Introduction

Members of the phylum *Chlamydiae* form a phylogenetically isolated group of bacteria. It includes the family *Chlamydiaceae*, which are pathogenic bacteria infecting a wide range of Vertebrates, as well as symbionts of free-living amoebae and other eukaryotic hosts, often referred to as environmental chlamydiae (Horn, 2008). The species that are pathogenic for humans include *Chlamydia trachomatis*, an agent of acute and chronic genital and ocular infection (Gerbase *et al*., 1998; Wright *et al*., 2008), and *Chlamydia pneumoniae*, responsible for approximately 10% of all community acquired pneumonia and 5% of bronchitis and sinusitis cases (Grayston *et al*., 1990). *C. pneumoniae* infections have been associated to a number of chronic inflammatory conditions such as atherosclerosis, adult-onset asthma and Alzheimer's disease (Blasi *et al*., 2009).

Members of the phylum share two characteristics: an obligate intracellular lifestyle and a unique biphasic developmental cycle (AbdelRahman and Belland, 2005). Infection starts with the attachment of the infectious form of the microorganism, the elementary body (EB), to a eukaryotic host cell. Upon attachment, intracellular signalling events lead to the internalization of the bacterium in a compartment called an inclusion. Importantly, the remainder of the developmental cycle takes place inside this compartment. During the early phase of this cycle, internalized EBs convert to non-infectious forms called reticulate bodies, which replicate in the inclusion. At the end of the developmental cycle, the bacteria differentiate back into EBs that are released to the extracellular space.

Entry of *Chlamydia* is accompanied by a local reorganization of the actin cytoskeleton, which is orchestrated by small GTPases of the Rho family and, at least in some

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species, of the Arf family (Carabeo *et al*., 2004; Subtil *et al*., 2004; Balañá *et al*., 2005). Translocation of effector proteins through the type III secretion (T3S) machinery probably plays a prominent role in orchestrating cytoskeletal reorganization and bacterial internalization, although it is not excluded that signalling through membrane receptors upon bacterial attachment also occurs. Two proteins secreted at the entry step have been described. The protein TARP is present in all *Chlamydiaceae*, and participates to the remodelling of the actin cytoskeleton (Clifton *et al*., 2004; Jewett *et al*., 2006; reviewed in Scidmore, 2011). The protein CT694 is only present in *C. trachomatis* and *C. muridarum*. It is also secreted upon invasion and interacts with host AHNAK, suggesting another link between early *Chlamydia* effectors and regulation of the actin cytoskeleton (Hower *et al*., 2009). *Chlamydia* T3S machinery remains active beyond the entry step, to deliver proteins across the inclusion membrane throughout the *Chlamydia* developmental cycle. Effector proteins have been implicated in a variety of functions including interactions with the host intracellular trafficking, inhibition of apoptosis and suppression of the NFκB pathway (Betts *et al*., 2009). In particular *C. trachomatis* secrete two proteins with deubiquitinating and deneddylating activities (Misaghi *et al*., 2006), which may be involved in this latter process (Le Negrate *et al*., 2008).

Throughout the bacterial kingdom, a prominent category of effector proteins is the one that modulates the host ubiquitin system (Collins and Brown, 2010; Kubori and Nagai, 2011). Ubiquitination is one of the most conserved post-translational modifications of proteins and is involved in many essential eukaryotic cell processes including protein degradation, transcriptional regulation, cell-cycle progression, and signalling (Haglund and Dikic, 2005). Many pathogenic bacteria use the host ubiquitin system to evade the immune system or to control the timing of their effector action. The recent recognition that ubiquitination is a conserved signal for selective autophagy might also explain why so many pathogens have acquired the ability to manipulate host ubiquitination capacity (Kraft *et al*., 2010). Upon reaching the cytosol, bacteria such as Group A *Streptococcus*, *Listeria* and *Salmonella*, are tagged with ubiquitin, which serves as a signal for targeting to degradation (Nakagawa *et al*., 2004; Perrin *et al*., 2004; Yoshikawa *et al*., 2009). Ubiquitin-interacting proteins such as NDP52, p62 and optineurin have recently been identified as autophagy adaptors that link ubiquitinated bacteria with the autophagosome-associated ubiquitin-like proteins (i.e. LC3/GABARAP proteins). For example, their ability to simultaneously bind ubiquitin and LC3 directs the ubiquitin-coated *Salmonella* to autophagosomes, thereby restricting bacterial growth (Thurston *et al*., 2009; Cemma *et al*., 2011; Wild *et al*., 2011). NDP52 and p62 are also recruited to cytosolic ubiquitinated *Listeria* and *Shigella* and target them to autophagic vesicles (Mostowy *et al*., 2011). Thus, ubiquitination appears as an innate defence mechanism for the cell, which, through the recruitment of adaptor proteins, directs cytosolic poly-ubiquitinated bacteria for autophagic destruction. Whether ubiquitination also takes place upon infection with a bacterium that remains in the vacuole, like *Chlamydia*, has not been investigated.

In this report, we show that ubiquitination occurs at the entry sites of the guinea pig adapted species *Chlamydia caviae*, a useful model for chlamydial entry (Subtil *et al*., 2004). However, local accumulation of ubiquitin is only observed during the first 15 min of infection. We provide evidence that the OTU domain-containing protein conserved in many *Chlamydia* species is a novel T3S effector responsible for the rapid loss of ubiquitin at the bacterial entry site.

Results

Local and transient accumulation of ubiquitinated proteins at Chlamydia *entry sites*

We examined ubiquitin distribution upon *Chlamydia* infection. We used *C. caviae* as a model because this species triggers intense actin polymerization upon entry in such a way that the bacterial entry sites are easily identified by staining the actin network using phalloidin (Subtil *et al*., 2004). The FK2 antibody was chosen to reveal ubiquitinated proteins, as it stains both mono- and poly-ubiquitinated proteins but not free ubiquitin. In uninfected cells, ubiquitinated proteins were homogenously distributed throughout the cell, and staining of the actin cytoskeleton was characteristic of resting cells (Fig. 1A). Ten minutes after infection with FITC-coupled bacteria, intense actin polymerization at the entry sites was observed, as previously described (Subtil *et al*., 2004). These sites were also enriched in ubiquitinated proteins in about 60% of the cells examined, indicating that local ubiquitination occurs at the entry sites. Ubiquitin enrichment covered a volume that was larger than the bacteria and coincided well with the actin foci, although the absence of perfect overlap between the two markers does not argue for ubiquitination of actin itself. Importantly, enrichment in ubiquitin was only transient since it was no longer visible 20 min after infection, concomitant with the depolymerization of actin that followed bacterial engulfment. Transient ubiquitination at bacterial entry sites was also observed when cells were transfected with HA-tagged ubiquitin prior to infection, and stained with anti-HA antibody (Fig. 1B). FK1 antibodies, which only stain poly-ubiquitinated proteins, also detected an enrichment of ubiquitin at the entry sites, suggesting that at least part of the signal comes from poly- rather than mono-ubiquitination (Fig. S1).

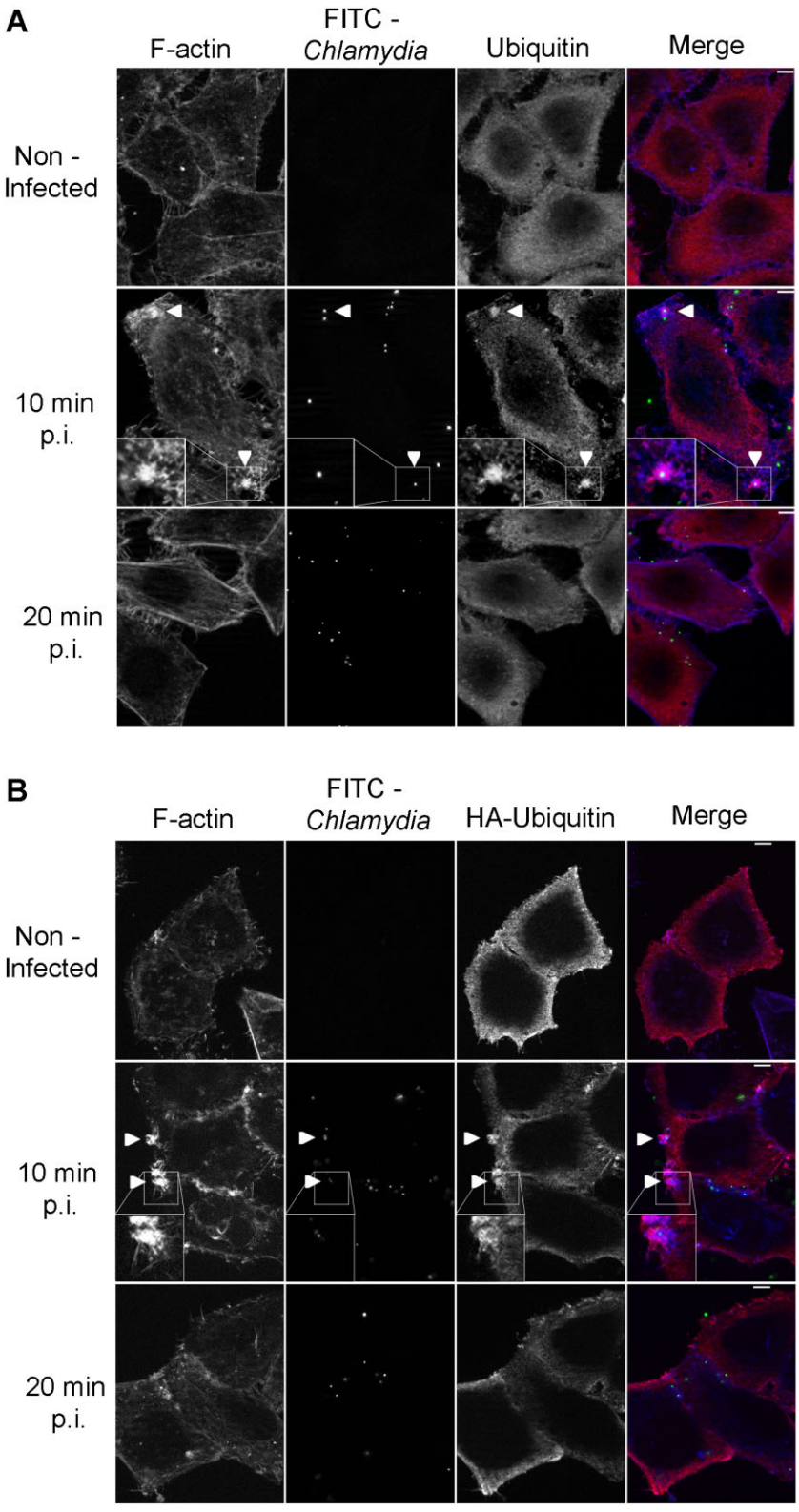

Fig. 1. Transient accumulation of ubiquitinated proteins at bacterial entry sites.
A. Infection was synchronized by centrifuging the FITC-coupled bacteria with the cells on coverslips at room temperature, before a 37°C incubation for the indicated periods of time and fixation with PFA. Cells were permeabilized, and ubiquitinated proteins were stained using FK2 antibody (third column), F-actin was stained with Alexa-350-conjugated phalloidin (first column), bacteria appear in the green channel (second column). Examples of areas of intense actin polymerization, which correspond to bacterial entry sites, are indicated with arrowheads. The inset shows a twofold magnification of an entry site.
B. Twenty-four hours prior to infection the cells were transfected with HA-tagged ubiquitin. Cells were infected as in (A), except that rat anti-HA antibodies (third column) were used to reveal the distribution of ubiquitin. Bar = 5 μM.

The OTU-like domain proteins of Chlamydia *are candidate deubiquitinases present in elementary bodies*

We hypothesized that the rapid disappearance of the ubiquitin-rich area at the entry site might result from the action of a bacterial activity, and we searched *Chlamydia* genomes for putative deubiquitinases. The *C. pneumoniae* protein CPn0483 had been predicted to belong to the OTU family of proteases (named after the ovarian tumour (*otu*) gene, a founding member of the family) (Makarova *et al*., 2000). Many members of the family were shown to possess deubiquitinating activity and they have been implicated in the regulation of immune pathways, including the NFκB pathway (Wertz *et al*., 2004; Kayagaki *et al*., 2007; Enesa *et al*., 2008). In addition to *C. pneumoniae*, the OTU-like domain was found in five other *Chlamydia* species, *C. caviae, C. abortus, C. felis, C. pecorum* and *C. psitttaci*, and not in *C. trachomatis* nor *C. muridarum*. Therefore, it is a gene specific for the formerly proposed *Chlamydophila* lineage, as opposed to *Chlamydia* lineage (that contains *C. trachomatis*) (Everett *et al*., 1999). An alignment of four chlamydial sequences was performed using the PROMAL software (Fig. S2). The proteins are similar, indicating that they are orthologues. Their N-terminus is predicted to form a non-globular domain with a hydrophobic segment followed by several polyproline stretches. The OTU-like domain lies downstream of the proline-rich region and is well conserved in all orthologues, in particular around the areas containing the putative catalytic residues (Fig. S2). No other features or domains were identified downstream of the OTU domain in a region of more than 600 amino acids, in which the sequences show an average of 25% identity. We further designate these proteins as *Chla*OTU.

We obtained rabbit polyclonal antibodies against *C. caviae Chla*OTU (CCA00261), which reacted with a protein of the expected molecular weight as assessed by Western blot on cell lysates infected for different times (Fig. 2B). The specificity of the antibody was verified using cells transfected with a GFP-tagged *Chla*OTU construct. Also, no signal was visible in cells infected with *C. trachomatis*, which do not express *Chla*OTU (Fig. 2C). *Chla*OTU was only detected 29 h after infection suggesting that the protein might accumulate in the EBs, which form at the end of the *Chlamydia* developmental cycle. Indeed, *Chla*OTU was detected in a bacterial lysate made from purified EBs (Fig. 2C, right lane), confirming that the protein is present in the infectious particles. Based on the intensities of the signals detected by Western blot, it is likely that the protein is not abundant in EBs, making its detection by immunofluorescence very challenging. In any case, the antibodies were unable to detect GFP-tagged CCA00261 when transiently expressed by transfection in HeLa cells, although the protein was expressed (data not shown). Therefore, we were not able to assess the localization of *Chla*OTU in infection by immunofluorescence. Nonetheless, its kinetics of expression is consistent with storage in EBs and with an early function in the infectious cycle.

The N-terminal segment of CPn0483 and CCA00261 are recognized as T3S substrates

Besides chlamydiae, the OTU-like domain is only found in eukaryotic cells and viruses (Makarova *et al*., 2000, and our analysis on current genomic data). It is therefore likely that the *chlaOTU* gene codes for a protein translocated in the host cell during infection. T3S is the main process, although not the exclusive one, by which chlamydial proteins are translocated into the host cell. The N-terminal segment of T3S substrates serves as secretion signal and, based on this property, we have set up a heterologous secretion assay using *Shigella flexneri* to identify chlamydial effectors (Subtil *et al*., 2001). To test the hypothesis that CPn0483 and CCA00261 might be T3S effector proteins, we fused their N-terminal sequence to the reporter protein calmodulin-dependent adenylate cyclase (Cya). The chimeric proteins were then expressed in *S. flexneri ipaB* (constitutive T3S) or *mxiD* (deficient in T3S) strains. When we analysed the culture supernatant versus the bacterial pellet, we found the chimeras in the supernatant of *ipaB* cultures (Fig. 2D). The same expression pattern was observed for the endogenous T3S substrate of *Shigella*, IpaD. Conversely, we found the cAMP receptor protein (CRP), a non-secreted protein, exclusively in the bacterial pellet excluding the possibility of non-specific leaking into the supernatant. This result demonstrates that the chimeras were secreted to the culture supernatant by the *ipaB* strain. Furthermore, the chimeras were found primarily in the bacterial pellet when expressed in the *mxiD* strain, indicating that secretion occurs via T3S. There is little conservation in the N-terminal extremity of the two orthologues (Fig. 2D). Therefore, the finding that both sequences function as T3S signals is highly indicative that the corresponding proteins are T3S effectors.

The C-terminal domain of Chla*OTU binds NDP52*

To investigate the function of the CPn0483 family of proteins we searched for host interacting proteins by yeast two-hybrid using as bait a fragment of CPn0483 without the first 69 amino acids (Δ69CPn0483). NDP52 was recovered five times in the screen (out of a total of 279 clones corresponding to 37 different proteins), with at least three independent clones. This result was extremely intriguing considering that NDP52 is known to bind ubiquitin (Thurston *et al*., 2009). The minimal domain of NDP52 interacting with Δ69CPn0483 encompassed amino acids

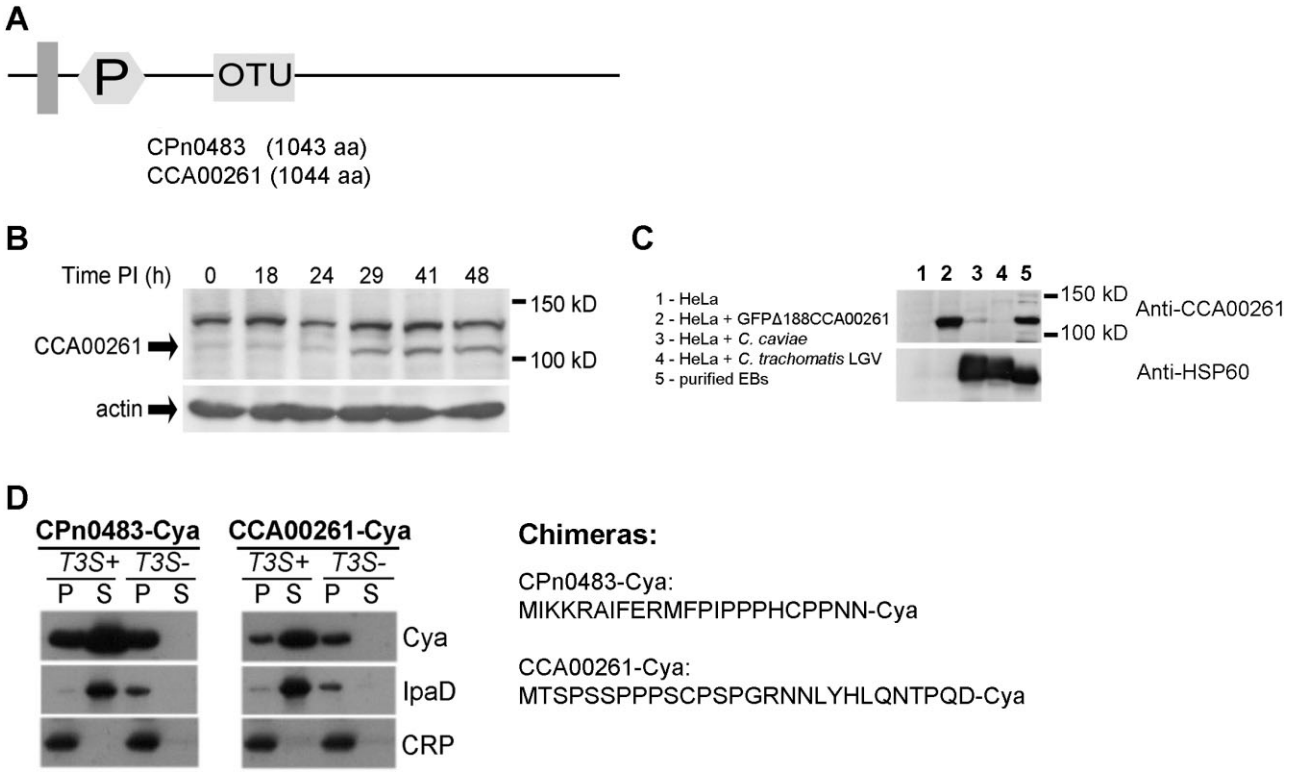

Fig. 2. *Chlamydia* proteins containing an OTU-like domain are present in infectious particles and are T3S substrates.
A. Schematic representation of CPn0483. Depicted domains are the hydrophobic region (grey rectangle), the proline-rich region (P) and the ovarian tumour-like domain (OTU). See also Fig. S3 for details.
B. Kinetics of CCA00261 expression in HeLa cells. Cells were infected for the indicated periods of time, lysed and whole-cell lysates were analysed by Western blot with anti-CCA00261 antibodies. Antibodies against actin were used as loading control. An experiment representative of two is shown.
C. Specificity of the antibody. HeLa cells were transfected with a construct coding for a GFP-tagged version of CCA00261 with a N-terminal truncation of 188 amino acids (expected molecular weight: 124 kDa) or infected for 30 h with *C. caviae* or *C. trachomatis* LGV, as indicated. The right lane was loaded with bacteria purified on a density gradient (EB). Antibodies against Hsp60 were used to show that the bacterial load in infected sample were similar. Note that the CCA00261 signal seen in *C. caviae*-infected cells is absent from *C. trachomatis*-infected cells, which do not have *Chla*OTU.
D. Chimeras were constructed encoding the indicated N-terminal sequence of each protein upstream of the reporter Cya. Chimeras were transformed into *Shigella ipaB* (T3S +) and *mxiD* (T3S –) strains. Exponential cultures were subfractionated into culture supernatant (S) and bacterial pellet (P). Samples were resolved by SDS-PAGE and analysed by immunoblotting against Cya, IpaD and CRP. An experiment representative of two is shown.

92–409 of the protein. In addition, three hits were further investigated, SIAH1, ANAPC5 and TRIAD3A, because they correspond to ubiquitin conjugating E3 ligases, a category of proteins often found to interact with deubiquitinases. Interaction between Δ69CPn0483 and these candidate proteins was tested by co-immunoprecipitation with lysates of HEK293T cells overexpressing each of the proteins with an N-terminal GFP tag and flag-tagged Δ69CPn0483. NDP52 co-fractionated with Δ69CPn0483 in the immunoprecipitated fraction while the three E3 ligases tested did not (Fig. 3A, data not shown for SIAH1 and ANAPC5). CT671, a *C. trachomatis* T3S substrate (Subtil *et al.*, 2005), served as a negative control and was not observed in the immunoprecipitated fraction. We then performed similar experiments using the *C. caviae* homologue of CPn0483. CCA00261 deleted of its first 52 amino acids co-immunoprecipitated with full-length NDP52 and with the fragment recovered in the two-hybrid screen GST-NDP52(92–409) (Fig. 3B). This result demonstrates that the interaction between *Chla*OTU and NDP52 was conserved during evolution, implicating that NDP52 is a target of the bacterial effector.

To map the regions of CPn0483 involved in the interaction with NDP52, we performed a GST pull-down. We cloned and purified the fragment of NDP52 identified to interact with CPn0483, with an N-terminal GST tag and used it to pull down interacting proteins from HEK293T cell lysates expressing different fragments of CPn0483. Δ192CPn0483 and Δ466CPn0483 were efficiently pulled down by GST-NDP52(92–409), while CPn0483-OTU was not (Fig. 3C). GST alone did not pull down any of these

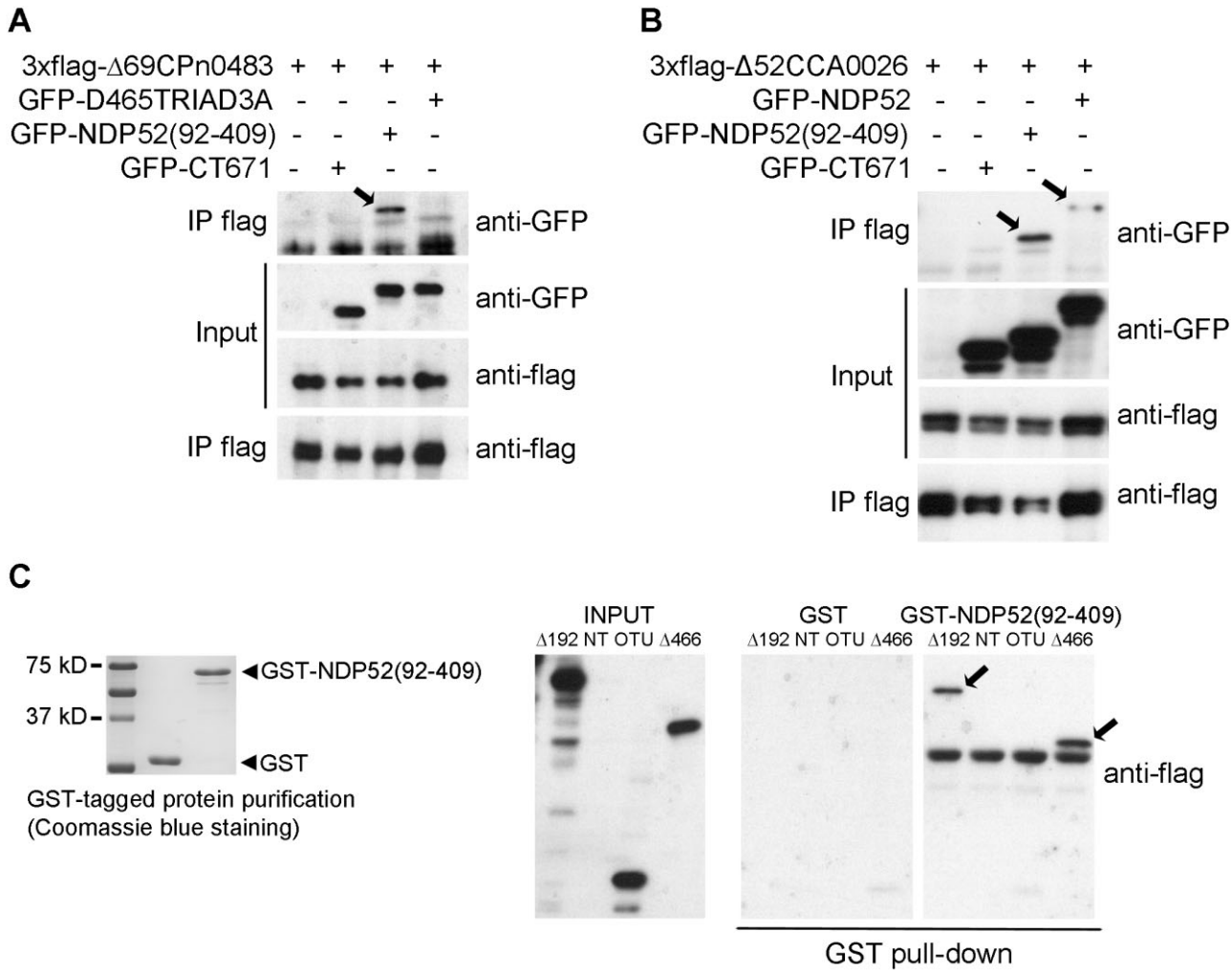

Fig. 3. *Chla*OTU binds to NDP52.
A. HEK293T cells overexpressing 3xflag-tagged Δ69CPn0483 alone or with one of the indicated GFP-tagged constructs were lysed and immunoprecipitation was performed using antibody anti-flag. Immunoblots were carried out using anti-GFP antibodies (top panel). The same membranes were then blotted with anti-flag antibodies (bottom panel). GFP-CT671 is a non-related GFP-tagged chlamydial protein used as negative control. Black arrows point to the co-immunoprecipitated protein.
B. Same experiment performed using HEK293T cells overexpressing 3xflag-Δ52CCA00261 and GFP-NDP52. CCA00261 co-immunoprecipitated both with full-length NDP52 and with the minimal interaction fragment found in the two-hybrid screen.
C. Equal amounts of HEK293T soluble extracts of cells overexpressing 3xflag-tagged fragments of CPn0483 were incubated with 5 μg of purified GST-NDP52(92–409) or GST only. After incubation with glutathione beads, the beads were washed and bound proteins were eluted with SDS-PAGE sample buffer. Samples were then subjected to SDS-PAGE and immunoblotted with anti-flag. Left panel shows a Coomassie blue gel staining of the purified GST proteins used as input in the pull-down. Black arrows highlight the CPn0483 fragments which bind NDP52. Δ192: 3xflag-Δ192CPn0483; OTU: 3xflag-Δ192CPn0483Δ570; Δ466: 3xflag-Δ466CPn0483; NT: non-transfected cells.
In each panel, an experiment representative of two is shown.

constructs. These observations confirm that CPn0483 interacts with NDP52 and mapped the interaction to the C-terminal domain, downstream of the OTU-like domain.

NDP52 is transiently recruited to the bacterial entry site and is dispensable for invasion

Our observation that *Chla*OTU accumulated in the infectious particles suggested that the effector might be secreted at the invasion step. Therefore we investigated the distribution of its interactor NDP52 during these events. A faint enrichment of NDP52 at the invasion sites could be observed using antibodies against the endogenous protein (data not shown). When we overexpressed flag-tagged NDP52 prior to infection, we observed a clear accumulation of the protein at the invasion sites 10 min after infection (Fig. 4A). This accumulation did not depend on the C-terminal ubiquitin-binding domain of NDP52, since a mutant lacking this domain (NDP52ΔZnF) was still recruited (Fig. 4B and C). NDP52 recruitment at the entry

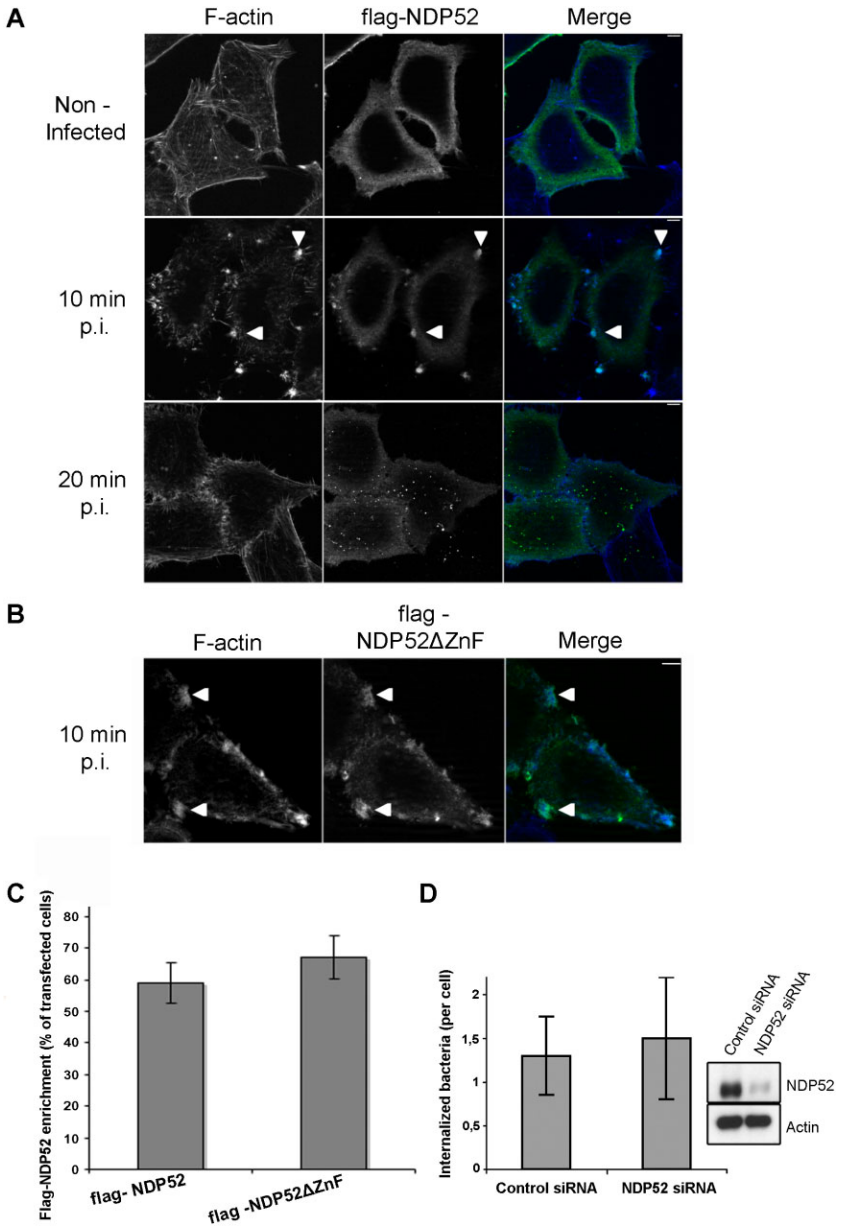

Fig. 4. NDP52 is transiently recruited at bacterial entry sites and is dispensable for invasion.
A. Twenty-four hours prior to infection the cells were transfected with flag-tagged NDP52. Cells were infected with bacteria by centrifugation at room temperature and incubated at 37°C for the indicated periods of time. Cells were permeabilized and ubiquitinated proteins were stained using anti-flag antibody (middle column), F-actin was stained with Alexa-350-conjugated phalloidin (left column). Examples of areas of intense actin polymerization, which correspond to bacterial entry sites, are indicated with arrowheads. Bar = 5 μM.
B. Same experiment performed on cells transfected with a NDP52 C-terminal deletion construct, removing the ubiquitin-binding Zn finger.
C. Quantification of NDP52 recruitment. Transfected cells were infected for 10 min, fixed and stained for NDP52 and actin. Number of cells in which NDP52 was observed in foci, relative to the total transfected population, was scored in duplicates and in two independent experiments ($n > 50$ cells for each score, error bar = SD).
D. Cells transfected with siRNA for NDP52 or control siRNA and bacterial entry assays were performed 48 h later, as described in Subtil *et al.* (2004). Intracellular bacteria were manually counted in transfected cells in nine randomly chosen fields and divided by the number of cells examined in that field (> 60 cells counted, error bar = SD). One experiment representative of two is shown. The right panel shows a Western blot against whole-cell lysates using anti-NDP52 antibodies, to estimate the extent of NDP52 extinction. Equal protein quantities were loaded in each lane, actin serves as a loading control.

site was transient and was no longer visible 20 min after infection. To test if NDP52 was needed for bacterial entry and development we used siRNA against NDP52 to deplete this protein before infecting the cells. Depletion of NDP52 did not affect *C. caviae* or *C. pneumoniae* inclusion growth and the resulting progeny was as infectious as progeny obtained from control cells (data not shown). To exclude a possible marginal need for NDP52 at the entry step, which could go unnoticed with a read-out on inclusion size or progeny, we also measured *C. caviae* entry in NDP52-depleted cells (Fig. 4D). Bacterial entry proceeded normally in NDP52-depleted cells, compared with control cells. Altogether, these data show that NDP52 is transiently recruited at the entry step, concomitant with the accumulation of ubiquitinated proteins. It appears to be dispensable for the internalization of the bacteria and for their subsequent development.

CPn0483 OTU-like domain binds ubiquitin

Ubiquitin-derived probes directed at enzyme active sites have been developed to identify novel deubiquitinases (Borodovsky *et al.*, 2002). They contain thiol-reactive groups in lieu of the C-terminus glycine residue of ubiquitin and include Michael acceptor groups (such as vinyl methyl ester – HAUb-VME) or alkylhalide groups (such as a bromoethylamine group – HAUb-Br_2). The electrophilic groups from the ubiquitin-derived probes are capable

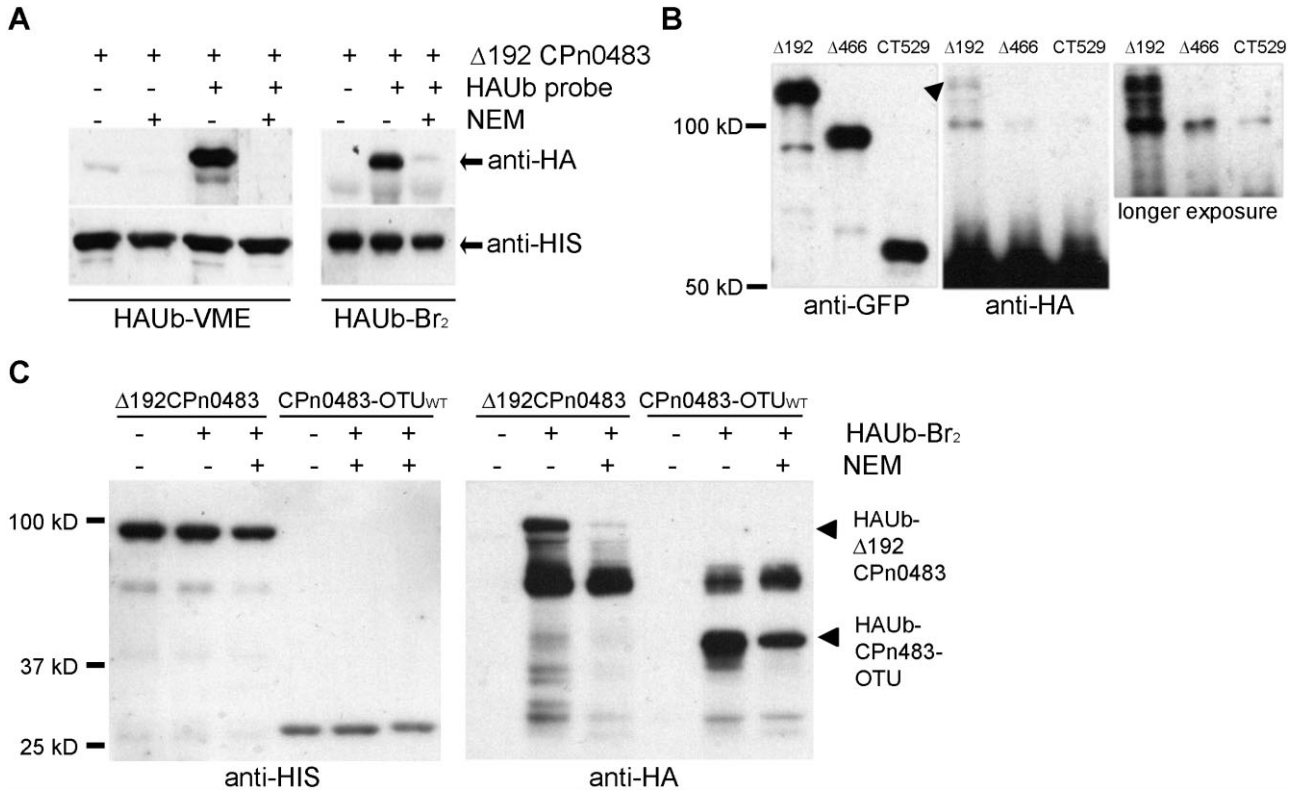

Fig. 5. CPn0483 binds covalently to ubiquitin-derived probes.
A. Purified His-tagged Δ192CPn0483 was incubated for 1 h at 37°C with 0.6 μg of HAUb-VME or HAUb-Br2, in the presence or absence of *N*-ethymaleimide (NEM). Protein was subsequently subjected to 8% SDS-PAGE and immunoblotted successively with anti-HA (upper panel) and anti-HIS (bottom panel) antibodies.
B. GFP-tagged proteins were expressed in HEK293T cells and immunoprecipitated with anti-GFP. Immunoprecipitates were incubated with 0.6 μg of HAUb-Br2 for 1 h at 37°C, subjected to 10% SDS-PAGE and immunoblotted with antibodies anti-GFP (left panel) and anti-HA (right panel). Δ192 designates Δ192CPn0483; Δ466, Δ466CPn0483; the *C. trachomatis* effector CT529 was used as a negative control. Arrowhead points to HA-Ub linked to Δ192CPn0483. Note that in addition, the anti-HA antibody detected a protein around 100 kD, in all samples (including the negative control), which correspond to a contaminating signal.
C. Purified 6xHIS-tagged Δ192CPn0483 and CPn0483-OTU were incubated with HAUb-Br2, in the presence or absence of NEM. Arrowheads point to the proteins linked to the HA-tagged probe.
An experiment representative of two is shown.

of reacting with the active-site cysteine of a target deubiquitinase, forming an irreversible thioester adduct. Alkyl halide-containing probes display more restricted activity than Michael acceptors, and the HAUb-Br_2 probe was chosen because of its ability to bind covalently to the catalytic site of a ubiquitin hydrolase belonging to the OTU family of deubiquitinases (Borodovsky *et al.*, 2002). Each probe is approximately 10 kDa and is tagged with a haemagglutinin (HA) epitope at its N-terminus to facilitate detection by immunoblot.

Full-length CPn0483 was expressed at a very low level (data not shown), possibly because of the poly-Proline sequence in its N-terminal part. We successfully expressed and purified from *Escherichia coli* a protein truncated of its first 192 amino acids (Δ192CPn0483, Fig. S3) as well as a shorter protein encompassing mainly the OTU-like domain (Δ192CPn0483Δ570, referred to as CPn0483-OTU). 6xHis-tagged Δ192CPn0483 was incubated with either HAUb-VME or HAUb-Br_2 for 1 h at 37°C. The samples were resolved by SDS-PAGE and immunoblotted with anti-HA, which resulted in the detection of an HA-specific band at 100 kDa, the expected molecular weight of Δ192CPn0483 protein (90 kDa) conjugated with the probe (10 kDa) (Fig. 5A). Labelling of Δ192CPn0483 with either probe was blocked by pre-incubation of the protein with the alkylating agent *N*-ethylmaleimide (NEM), which is consistent with the requirement of an active-site cysteine for catalysis and, therefore, linkage to the ubiquitin-based probe (Wilkinson, 1997).

The reactivity of HAUb-Br2 was further tested against the GFP-tagged version of CPn0483 produced in transfected HEK293T cells. Fragments of CPn0483 with (Δ192CPn0483) or without (Δ466CPn0483) the OTU domain where immunoprecipitated and incubated with HAUb-Br2. Probing the immunoprecipitates with anti-HA

antibody revealed a HA-band at the expected molecular weight for Δ192CPn0483. In contrast, the ubiquitin-derived probe did not bind the fragment without the OTU domain (Δ466CPn0483) nor GFP-tagged CT529, a non-related chlamydial protein used as a negative control (Fig. 5B). To demonstrate that the probe interacted with CPn0483 OTU-like domain, we repeated *in vitro* binding assays on the His-tagged CPn0483-OTU fragment. HaUb-Br2 probed reacted with CPn0483-OTU as it did with Δ192CPn0483, confirming that the OTU-like domain binds ubiquitin (Fig. 5C). Note that the absence of shift of the CPn0483-OTU fragment towards an 10-kD higher form (anti-HIS blot, Fig. 5C) indicates that only a minor fraction of the CPn0483-OTU fragments reacted with the probe, suggesting that the *in vitro* reaction is rather inefficient.

Chla*OTU functions as a deubiquitinase at the invasion step*

We next examined the ability of *Chla*OTU to cleave branched polyubiquitin chains. We expressed *C. caviae Chla*OTU with an N-terminal GFP tag in HeLa cells (a protein deleted from the first 188 amino acids was used to enhance expression). The ability for this construct to bind the HAUb-Br2 probe, similar to what we had observed with the *C. pneumoniae* homologue, was verified on protein immunoprecipitated from cell lysates (Fig. S4A). A construct in which the predicted catalytic cysteine was mutated to alanine, GFP-Δ188CCA00261C286A, did not react with the probe. In a second set of experiments, the protein was affinity-purified using antibodies against GFP and incubated with K48- or K63-linked multi-ubiquitin chains. Reaction products were separated by SDS-polyacrylamide gel electrophoresis, transferred on a membrane and immunoblotted with anti-ubiquitin antibody. Isopeptidase T, a ubiquitin-specific protease, served as a positive control. GFP-Δ188CCA00261, but not GFP, cleaved K48- and K63-linked chains. The catalytic mutant showed no activity (Fig. 6A). This experiment demonstrates that the OTU domain of *Chla*OTU functions as a deubiquitinase.

The two *Chla*OTU binding partners that we have identified, NDP52 and ubiquitin, are enriched at the chlamydial invasion sites, strongly indicating that *Chla*OTU secretion takes place at this step of the developmental cycle. We therefore examined the effect of the heterologous expression of *Chla*OTU, through transfection, on ubiquitin accumulation during entry. Transfection of GFP-tagged *Chla*OTU, or of its catalytic mutant, did not affect the overall level of ubiquitination in the cell, measured on cell lysates probed with anti-ubiquitin (P4D1) antibody (Fig. S4B). Cells expressing either GFP as control or the GFP-tagged *Chla*OTU constructs (wild type or catalytic mutant) were infected for 10 min, fixed and stained for ubiquitin. We observed that the GFP-*Chla*OTU constructs were recruited to the invasion sites, supporting the hypothesis that they play a role at this step. Accumulation of ubiquitin was clearly enhanced in the cells expressing the catalytic mutant of *Chla*OTU compared with cells expressing the wild-type form (Fig. 6B). A similar observation was made when HA-tagged ubiquitin was expressed together with the GFP-tagged constructs, and anti-HA antibodies used for staining (Fig. 6C). The number of cells in which ubiquitin enrichment was observed at the entry sites 10 min post infection was significantly increased in cells expressing the catalytic mutant of *Chla*OTU compared with GFP, and significantly decreased in cells expressing the functional deubiquitinase (Fig. 6D). Figure 6B and C shows examples of Δ188CCA00261-expressing cells in which the entry sites are not enriched in ubiquitin, in contrast to what is observed in cells expressing the catalytic mutant. Thirty minutes after infection ubiquitin was cleared even in the cells expressing the catalytic mutant of *Chla*OTU, and the percentage of infected cells measured 24 h after infection was identical for all constructs (data not shown). These observations suggest that the catalytic mutant only delayed deubiquitination at the entry site, which eventually took place, likely through the activity of the endogenous bacterial deubiquitinase.

Discussion

As obligate intracellular bacteria, chlamydiae use a significant portion of their genomes (at least 10%) to produce proteins directly targeting host activities. Given the difficulty of genetically manipulating this bacterium, in only a handful of cases has the function of effector proteins been studied. Here, we report the identification of a novel effector protein, which we designated as *Chla*OTU. This protein is well conserved in several *Chlamydia* strains, with the noteworthy exception of *C. trachomatis* and *C. muridarum*. The quality of our antibodies and the low abundance of *Chla*OTU precluded direct demonstration of the secretion of this protein during infection. However, we provide compelling evidence for the secretion of this protein: (i) the N-terminal segments of *C. pneumoniae* and *C. caviae Chla*OTU function as T3S signals. Very importantly, the conservation of the N-terminal extremity of the two orthologues is very low. Therefore, finding that both sequences function as T3S signals is highly indicative that the corresponding proteins are T3S effectors. (ii) *Chla*OTU binds to NDP52, a host protein. (iii) *Chla*OTU exhibits deubiquitinase activity, i.e. activity against a substrate only present in the host.

Chlamydia effectors can be secreted during the entry step, across the plasma membrane, or later in the developmental cycle, across the inclusion membrane. Our data strongly indicate that *Chla*OTU secretion starts very early

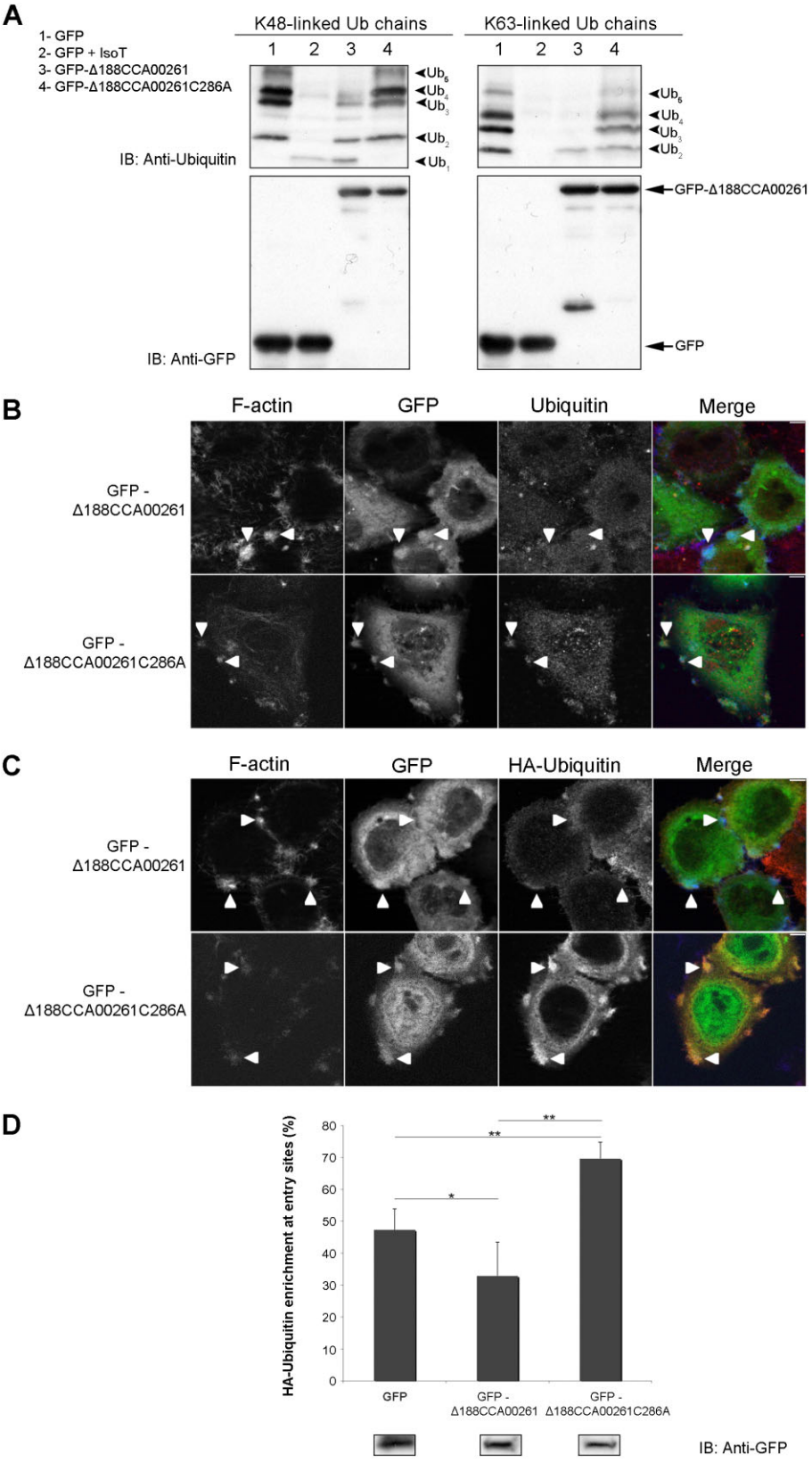

Fig. 6. Overexpression of *Chla*OTU induces clearance of ubiquitin from the entry sites.
A. GFP-linked proteins, immunoprecipitated from cells expressing GFP, GFP-Δ188CCA00261 or GFP-Δ188CCA00261C286A were incubated with K48- or K63-linked multi-ubiquitin chains overnight at room temperature. The chains were then separated by SDS-PAGE, transferred to a PVDF membrane and labelled with anti-ubiquitin antibody P4D1 (top blot). Note the disappearance of chains incubated with GFP-Δ188CCA00261 or with GFP in the presence of Isopeptidase T (IsoT), which serves as a positive control, and not with the catalytic mutant C286A. Monomers of ubiquitin are not well recognized by this antibody, explaining the relatively low signal for mono-ubiquitin (Ub1). The immunoprecipitated GFP-tagged proteins were run on a separate gel and immunoblotted with anti-GFP antibodies (bottom blot). The experiment is representative of two.
B. Cells were transfected with GFP-Δ188CCA00261 (top row) or GFP-Δ188CCA00261C286A (bottom row). Twenty-four hours later, cells were infected for 10 min at 37°C, fixed, permeabilized and ubiquitinated proteins were stained using FK2 antibody (third column). Alexa-350-conjugated phalloidin stains F-actin (first column), GFP-tagged constructs were visualized in the green channel (second column). Examples of areas of intense actin polymerization, which correspond to bacterial entry sites, are indicated with arrowheads. In GFP-Δ188CCA00261-overexpressing cells, some of these areas show no accumulation of ubiquitin. Bar = 5 μM.
C. Same experiment as in (B), except that the cells were co-transfected with HA-tagged ubiquitin together with the GFP-tagged constructs. Anti-HA antibodies (third column) were used to reveal the distribution of ubiquitin.
D. Quantification of the experiment shown in (C). The percentage of cells in which HA-ubiquitin was observed in foci, relative to the total number of transfected cells analysed, was calculated ($n > 60$ cells for each score). Values are the means (± SD) of three independent experiments, $^{*}P < 0.05$, $^{**}P < 0.01$. The level of expression of each construct was quantified in parallel by Western blot on cell lysates normalized for protein content, using anti-GFP antibodies, and was found to be similar (blots below the histogram).

during invasion. First, the protein was detected in elementary bodies purified on a density gradient, demonstrating that *Chla*OTU is present in the infectious particles. It is generally assumed that most effectors stored in the infectious particles play a role early in infection. During the entry step, bacterial protein synthesis is absent and only effectors stored in the bacteria during the previous developmental cycle are available. Second, as discussed below, we observed a modification on the distribution of *Chla*OTU-binding protein NDP52 as early as 10 min after infection, and clearance of ubiquitinated proteins 10 min later, which strongly suggests that *Chla*OTU reaches the host cytosol within the first minutes of infection.

Using ubiquitin-derived probes, we showed that *Chla*OTU binds ubiquitin, and more specifically through its OTU-like domain. We confirmed the ability of *Chla*OTU to cleave multi-ubiquitin chains *in vitro* using *Chla*OTU purified from transfected cells. Interestingly, we had initially performed the *in vitro* assay using GST-*Chla*OTU (from *C. pneumoniae*) purified from *E. coli*, with negative results (data not shown). Similar experimental conditions had been tested, and the difference could be due to a better purification yield in the mammalian expression system. Alternatively, a host cofactor and/or maturation step might be needed to trigger *Chla*OTU activity, which would be missing in the bacterial expression system. Although we could not confirm it by co-immunoprecipitation experiments, it is interesting to note that the two-hybrid screen identified three E3-ligases as potential *Chla*OTU-binding proteins. Deubiquitinases and E3 ligases are often associated in complexes, and further work will be needed to test whether such is the case here.

Transcriptomics data indicate that *chlaOTU* is expressed very early (Maurer *et al*., 2007), suggesting that after the initial stock of protein is secreted in the early stage of infection, neosynthesis might take over to ensure maintenance of the deubiquitinase activity throughout the developmental cycle. Our failure to detect the protein until mid-phase of the developmental cycle by Western blot does not speak against this possibility: secreted *Chla*OTU might be rapidly turned over, never accumulating to detectable levels until its secretion is turned off in the bacteria, when converting to elementary bodies.

In addition to ubiquitin, we identified the host protein NDP52 as a *Chla*OTU-binding protein. Interaction between the two proteins was observed using different approaches (two-hybrid in yeast, co-immunoprecipitation, and pull-down in transfected cells). We showed that the region of *C. pneumoniae Chla*OTU involved in the interaction was downstream of the OTU-like domain, indicating that the protein might be able to contact ubiquitin and NDP52 simultaneously. Importantly, we showed that the ability for *Chla*OTU to bind NDP52 was conserved in *C. caviae*, arguing for a central role in this effector's function. NDP52 is a cytosolic protein, enriched at the *trans* side of the Golgi complex and is also associated to vesicles distributed throughout the cell (Morriswood *et al*., 2007). Among other roles, NDP52 has been implicated in vesicular transport out of the *Golgi* apparatus. Given that *Chlamydia* hijack part of the vesicular traffic from the *Golgi* apparatus (Hackstadt *et al*., 1996), this could hint to a role for NDP52 in membrane trafficking to the inclusion. However, we did not observe recruitment of NDP52 to the inclusion in *C. pneumoniae*- or *C. caviae*-infected cells (data not shown), and we found that depletion of NDP52 did not affect bacterial development. Finally, we cannot exclude the possibility that NDP52 recruitment to the invasion sites plays a role in the regulation of the actin-mediated internalization process, and that the redundancy of mechanisms at work in this process explains why NDP52 depletion has no effect on invasion.

One of the new findings of this work is that ubiquitination occurs at *Chlamydia* invasion sites. Whether this signal is due to local ubiquitination [of host or bacterial protein(s)], or to the recruitment of ubiquitinated protein(s) remains to be determined. Given the current absence of tools to prevent the ubiquitination/deubiquitination cycle (even expression of the catalytic mutant only delays deubiquitination), its physiological role will be difficult to address experimentally. One attractive hypothesis is that, like in other infectious processes, the host cell responds to *Chlamydia* invasion by a local ubiquitination, meant to alert the autophagy machinery. Secretion of *Chla*OTU would counteract this defence mechanism, by clearing ubiquitin. The ability of *Chla*OTU to bind NDP52 supports such a role for *Chla*OTU, since NDP52 has recently been recognized as an adaptor protein linking ubiquitinated cargo and autophagosomal membranes. We did not recover clones coding for other autophagy adaptors such as p62 or optineurin in the two-hybrid screen using *Chla*OTU as a bait, and p62 did not co-immunoprecipitate with GFPD188CCA00261 (data not shown), suggesting that *Chla*OTU targets NDP52 and not other autophagy adaptors. A NDP52 construct lacking the C-terminal ubiquitin-binding domain is still recruited to the invasion sites, indicating that local ubiquitination is not the only trigger for NDP52 recruitment to these sites. The distribution of ubiquitin and NDP52 is somewhat diffuse around the invasion sites, similar to the distribution of polymerized actin, while *Chla*OTU distribution might be restricted to the nascent vacuole, if its hydrophobic N-terminal domain serves as a transmembrane anchor. Whether ubiquitin and NDP52 form a transient scaffold around nascent inclusions, and what initiates its formation, remains to be investigated. One possibility is that by binding to the distal domain of *Chla*OTU, NDP52 brings the ubiquitinated substrates to the catalytic OTU domain, for deubiquitination. Another no mutually exclusive scenario is that by binding

NDP52, *Chla*OTU might mask LC3 binding sites in NDP52, thus preventing the recruitment of autophagosomal membranes. Indeed, the autophagy marker LC3 was not recruited to the entry sites (data not shown), indicating that the short-lived recruitment of ubiquitinated proteins to invasive bacteria was not sufficient to target the *Chlamydia*-containing vacuole to autophagic membranes.

In conclusion, we have identified a novel early type III effector, conserved in several *Chlamydia* strains, which displays deubiquitinase activity and binds NDP52. While *chla*OTU is absent from *C. trachomatis* and *C. muridarum* genomes, two proteins with deubiquitinating and deneddylating activities have been described in these species, which are absent from *C. pneumoniae* and *C. caviae* (Misaghi *et al*., 2006). It is quite remarkable that chlamydiae have acquired deubiquitinase activity at two occasions. It is tempting to speculate that *Chla*DUBs may fulfil *Chla*OTU functions in *C. trachomatis* infection. In support of this hypothesis, *Chla*DUB1 was found to be enriched in elementary bodies (Saka *et al*., 2011). The acquisition of deubiquitinase activities may represent a key step in the adaptation of pathogenic chlamydiae to novel niches.

Experimental procedures

Cell culture and Chlamydia *propagation*

HeLa 229 cells were cultured in Dulbecco's modified Eagle's medium with Glutamax (DMEM, Invitrogen) supplemented with 10% FCS. HEK293T cells (ATCC number CRL-11268) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% FCS and 100 U penicillin/100 μg of streptomycin (Invitrogen). *C. caviae* strain GPIC was obtained from Dr R. Rank (University of Arkansas) and was propagated in HeLa cells as described (Subtil *et al*., 2004). EBs were purified on discontinuous density gradient centrifugation in Renografin (Radiosélectan 76%, Schering, SA) (Scidmore, 2005).

Plasmids

Ub-HA plasmid was obtained from Franck Gesbert (Villejuif, France). Genomic DNA was isolated from *C. pneumoniae* and *C. caviae* using the RapidPrep Micro Genomic DNA isolation kit (Amershman Biosciences). attB-containing primers (Gateway, Invitrogen) specific to CPn0483, CCA00261, TRIAD3A, SIAHA, ANAPC5 and NDP52 were constructed by PCR and sequence verified (Table S1), allowing expression of the proteins with 6xHis or GST tags in bacteria, and 3xflag or GFP tags in mammalian cells. 3xflag conjugated NDP52 was amplified from cDNA made from HeLa cells, other mammalian genes were amplified on the clones obtained in the two-hybrid screen. All constructs were verified by sequencing. CCA00261 optimized for expression in mammalian cells was obtained from Genscript and cloned into pEGFPC2 (Clontech), truncating the first 188 amino acids (Δ188CCA00261, which correspond to the *C. caviae* homologue of Δ192CPn0483). The amino acid sequence was unchanged, and the optimization resulted in a significant increase in the expression levels. The catalytic mutant Δ188CCA00261C286A was constructed using the QuickChange Site-Directed Mutagenesis kit (Stratagene) following the manufacturer's instructions.

Immunofluorescence microscopy

HeLa cells seeded on coverslips were infected with *C. caviae* by centrifugation to synchronize entry as described (Subtil *et al*., 2004). In some experiments infectious particles coupled to FITC were used (Subtil *et al*., 2004).

Plates were incubated at 37°C for the indicated times, washed twice in PBS, and fixed in 4% paraformaldehyde (PFA), 120 mM sucrose in PBS for 20 min at room temperature, before permeabilization in 0.05% saponin. Endogenous ubiquitin was stained overnight at 4°C with antibodies against mono- and poly-ubiquitinated proteins (clone FK2, Enzo Lifesciences). In a second step, when indicated the actin stain Alexa-350 or Alexa-546 conjugated-phalloidin (Molecular Probes) was added to the secondary antibodies (Alexa488 or Alexa568-coupled anti-mouse antibody). In some experiments cells were transfected with plasmids expressing HA-tagged ubiquitin or 3xflag-tagged NDP52 the day before infection with JetPrime™ reagent (PolyPlus Transfection, France). Ubiquitin-HA was revealed using Rat anti-HA antibody (3F10, Roche), followed with TRITC-coupled anti-Rat secondary antibody (Jackson ImmunoResearch); 3xflag-tagged NDP52 was stained with anti-flag antibody (anti-flag M2, Sigma) followed with Alexa-488 coupled anti-mouse antibody. Images were acquired on an Axio observer Z1 microscope equipped with an ApoTome module (Zeiss, Germany) and a 63× Apochromat lens. Pictures were taken with a Coolsnap HQ camera (Photometrics, Tucson, AZ) using the software Axiovision.

Quantification of ubiquitin or NDP52 recruitment to the entry sites

Random fields of cells infected for 10 min stained for actin and ubiquitin or NDP52 as described above were acquired. The number of cells in which ubiquitin or NDP52 was enriched in the actin foci (evaluated by eye examination) was quantified, and expressed relative to the number of cells examined ($n > 200$).

Bioinformatics

CPn0483 amino acid sequence was examined using different EXPASY tools (PROSITE, SMART, SIGNALP 3.0 and TMHMM 2.0). Chlamydial protein sequences containing an OTU domain were obtained with HMMER search, with the Pfam database OTU-like cysteine protease model. OTU-containing sequences were aligned using the multiple sequence alignment program PROMALS.

Shigella *heterologous secretion assay*

The 5′ part of *cpn0483* and *cca00261*, including about 30 nucleotides located upstream from the translation start sites and the first 23 or 28 codons respectively, were amplified by PCR and

cloned in the puc19cya vector (Table S1). Analysis of secreted proteins was performed as described previously (Subtil *et al.*, 2001).

Electrophilic labelling and detection

For each labelling reaction, around 5 μg of purified 6xHIS-tagged protein in purification elution buffer was incubated with 0.6 μg of probe in a total volume of 60 μl of homogenization buffer (50 mM Tris pH 7.5, 5 mM $MgCl_2$, 0.5 mM EDTA, 2 mM DTT, 2 mM ATP and 250 mM sucrose). For reactions involving NEM, protein was incubated at room temperature for 30 min with 10 mM NEM prior to addition of probe. Reactions were incubated for 60 min at room temperature. Samples were boiled in reducing sample buffer and separated by SDS-PAGE and immunoblotted with anti-HA.11 (Covance) or anti-HIS.

Yeast two-hybrid assay

The yeast two-hybrid assay was performed using CPn0483 (amino acids 70–1043, downstream of GAL4 DNA-binding domain) as bait to screen a human placenta cDNA library RP4 (Hybrigenics). A total of 155×10^6 interactions were analysed, 279 clones were processed.

Production of rabbit polyclonal antibody to CCA00261

6xHis-tagged Δ221CCA00261Δ606 were expressed in BL21DE3(pLysS). Bacteria were induced with 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) overnight at 18°C. The protein was purified in 6 M urea buffer and used as immunogen for the production of specific polyclonal antibodies in New Zealand White rabbits (Agro-Bio, La Ferté Saint-Aubin, France).

Production of 6xHIS-tagged and GST-tagged recombinant protein

Escherichia coli Rosetta-Gammi 2 (pLysS) (Novagen) was transformed with protein expression vectors (see above) and plated on Luria–Bertani (LB) plates containing 100 μg ml^{-1} ampicillin with or without 34 μg ml^{-1} chloramphenicol, 12.5 μg ml^{-1} tetracycline and 50 μg ml^{-1} streptomycin. Overnight cultures were inoculated into 500 ml LB broth containing 100 μg ml^{-1} ampicilin, incubated at 37°C until the optical density at 600 nm was 0.4–0.6, and then cooled on ice. The production of recombinant protein was initiated with the addition of 0.2 mM IPTG and cultures were incubated at 14°C overnight. Cultures were then centrifuged at 4000 *g* for 20 min, and pellets were lysed with a concentration factor of 60 with ice-cold lysis buffer (150 mM NaCl, 50 mM Tris pH 7.4, 10% glycerol, 1 mM TCEP and 10 mM imidazole) containing 0.5 mg ml^{-1} of lysozyme and 0.02 mg ml^{-1} DNase I. Lysates were centrifuged at 2.200 *g* for 10 min and then ultracentrifuged at 135 000 *g* in a TLA 100.3 rotor (Beckman). Recombinant protein was purified over Ni-NTA resin. The resin was washed with a step gradient of imidazole, and purified protein was released with elution buffer (150 mM NaCl, 50 mM Tris pH 7.4, 10% glycerol, 1 mM TCEP and 250 mM imidazole).

Expression of GST-tagged proteins was performed in *E. coli* BL21DE3(pLysS) in the same conditions, using 1× PBS containing 0.5 M NaCl, 2 mM DTT, 0.5 mg ml^{-1} lysozyme, 0.02 mg ml^{-1} DNase I, 1 mM PMSF and 1× protease inhibitors cocktail (Sigma) as lysis buffer. Insoluble material was removed by centrifugation at 100 000 *g* for 20 min at 4°C. Supernatants were rotated on a rocking platform for 1 h onto 250 μl glutathione sepharose (Amersham). Beads were collected by centrifugation at 300 *g* for 5 min at 4°C and washed two times with 1× PBS containing 0.5 M NaCl, 2 mM DTT with inhibitors. GST fusion proteins on glutathione agarose beads were eluted in buffer 50 mM Tris pH 7.4 and 150 mM NaCl and stored at 4°C for use in the pull-down assays.

Immunoprecipitation, immunodetection and pull-down experiments

HEK293T were grown to 80% confluency and transfected with the indicated plasmids using Lipofactamine2000 (Invitrogen). Confluent monolayers grown in 6 cm dishes were used for each immunoprecipitation. Cells were harvested at 24 h after transfection and lysed with NP40 lysis buffer (150 mM NaCl, 50 mM Tris pH 7.4, 5 mM MgCl2, 0.5 mM EDTA, 2 mM DTT and 0.5% NP40), rocking at 4°C for 1 h. Cell lysates were centrifuged at 12 000 *g* for 20 min at 4°C. Protein concentration of supernatants was established by Bradford assay. Supernatants were incubated with 1 μg of anti-flag or anti-GFP (Invitrogen) antibodies 2 h at 4°C on a rocking platform, followed by 1 h incubation with Sepharose beads (Amersham). Immunoprecipitates were collected by a brief centrifugation and were washed four times in lysis buffer and once in 1× PBS. The bound proteins were eluted with 1× SDS-PAGE sample buffer (63 mM Tris pH 7.4, 10% glycerol, 2% SDS, 0.01% bromophenol blue, 1% β-mercaptoethanol), boiled for 10 min and frozen.

Proteins were subjected to SDS-PAGE, transferred to Immobilon-P (PVDF) membranes and immunoblotted with the proper primary antibodies diluted in 1× PBS containing 5% milk and 0.01% Tween-20. Primary antibodies used were rabbit anti-HIS (Santa Cruz, sc-803), mouse anti-GFP (Santa Cruz), mouse anti-GST (Euromedex), mouse anti-HA.11 (Covance), followed by goat anti-mouse IgG-HRP (horseradish peroxidase) at 1:2000 dilution or goat anti-rabbit IgG-HRP at dilution 1:10 000 (GE Healthcare). Blots were developed using the Western Lightning Chemiluminescence Reagent (GE Healthcare).

For GST pull-downs, HEK293T were grown to 80% confluency and transfected with the indicated plasmids using Lipofactamine2000 (Invitrogen). Confluent monolayers grown in 6 cm dishes were used for each reaction. Cells were lysed in 500 μl of GST lysis buffer (20 mM Tris-HCl pH 8, 150 mM NaCl, 0.5 mM EDTA, 0.5% NP40 1 mM DTT, 1 mM PMSF and 1× protease inhibitor cocktail (Sigma) rocking at 4°C for 30 min. Cell lysates were centrifuged at 12 000 *g* for 20 min at 4°C and pre-cleaned twice with glutathione-agarose beads for 30 min at 4°C in a rocking platform. Equal amount of pre-cleaned supernatants were incubated with 50 μl of a 50% slurry of glutathione-agarose beads and 5 μg of purified GST or GST-NDP52 (amino acids 92–409) for 2 h at 4°C, on a rocking platform. After a brief centrifugation, beads were washed four times with cold GST lysis buffer. The bound proteins were then eluted with 1× SDS-PAGE sample buffer and treated as before.

Depletion of NDP52 by siRNA

Four million HeLa cells were transfected by electroporation (300 V, 500 μF) on day 1 with 40 pmol siNDP52#1 and

siNDP52#2 (Thurston *et al*., 2009) or ON-TARGET plus® control siRNA (Thermo Scientific Dharmacon). The following day cells were split and seeded in 24-well plates. On day 3, cells were infected for 30 min at 37°C and fixed in PFA. Extracellular bacteria were labelled in a first step, before cell permeabilization and staining of intracellular bacteria as described previously (Subtil *et al*., 2004). Cells from four wells were pooled for analysis of protein depletion by Western blot.

Deubiquitination assay

Four million HeLa cells were transfected with codon optimized GFP-Δ188CCA00261, GFP-Δ188CCA00261C286A or with GFP. One day after transfection GFP-tagged proteins were immunoprecipitated with anti-GFP antibodies as described above and the beads were cleaned in reaction buffer (TBS: 137 mM NaCl, 2.7 mM KCl, 25 mM Tris-Cl pH 7.4). Two hundred nanograms of either Lys-48- or Lys-63-linked multi-ubiquitin chains (Boston Biochem) were added to the beads in a 30 µl reaction volume. Purified Isopeptidase T (10 pmol; Boston Biochem) was used as a positive control. After incubation at room temperature overnight the samples were centrifuged and the supernatants were run on a 4–20% acrylamide gel (Bio-Rad), transferred onto a PVDF membrane and stained with anti-ubiquitin P4D1 antibody (Cell Signaling Technology) followed by goat anti-mouse IgG-HRP. The beads were run on a separate gel and blotted with anti-GFP antibody to check that equivalent amount of GFP-tagged proteins had been immunoprecipitated.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1. Local and transient accumulation of poly-ubiquitinated proteins at *Chlamydia* entry sites. Cells were infected or not with unlabelled *C. caviae* GPIC, following the procedure described in Fig. 1. Ubiquitinated proteins were stained with FK1 antibodies (Enzo Life Sciences), which stains poly-ubiquitinated proteins (middle column), followed with Alexa647-conjugated anti-IgM antibodies. F-actin was stained with Alexa4880-conjugated phalloidin (left column). The inset shows a twofold magnification of an entry site. Bar = 5 μM.

Fig. S2. Sequence alignment of the N-terminal portion of CPn0483 and its orthologues. The amino acid sequences of *C. pneumoniae* CPn0483, *C. abortus* CAB255, *C. caviae* CCA00261 and *C. felix* CF0747 were aligned using the PROMAL software. Only the first third of the proteins are shown, as no particular feature was identified in the rest of the sequence. Hydrophobic residues forming a putative transmembrane domain are highlighted in green, prolines of the proline-rich domain in red, and the OTU-like domain in yellow, with predicted catalytic residues in bold letter. A graphical representation of the similarity along the set of aligned sequences obtained by Plotcon (EMBOSS-package) is shown below.

Fig. S3. Production and purification of recombinant proteins.
A. Schematic representation of the constructs used in this study.
B. Purification of recombinant proteins used in this study.
6xHIS-tagged CPn0483 truncated of its first 192 amino acids (Δ192CPn0483) and the OTU domain region only (CPn0483-OTU) were expressed in *E. coli* Rosetta-Gami 2 (DE3)pLys (Novagen) and purified by affinity chromatography in a soluble form. Samples were run on SDS-PAGE and proteins stained with Coomassie blue. SF, soluble fraction; FL, flow-through; E, eluted fractions.

Fig. S4. *In vitro* assays using *C. caviae Chla*OTU.
A. GFP-tagged proteins were expressed in HeLa cells and immunoprecipitated with anti-GFP. Immunoprecipitates were incubated with 1 μg of HAUb-Br2 for 1 h at 37°C, subjected to 10% SDS-PAGE and immunoblotted with antibodies against GFP (left panel) and against HA (right panel). Arrowhead points to HA-Ub linked to GFPΔ188CCA00261. The mutation cysteine 286 to alanine abolished the ability for GFPΔ188CCA00261 to bind

the probe, strongly supporting the identification of cysteine 286 as a catalytic residue, as suggested by the alignment shown in Fig. S1.

B. GFP-tagged proteins were expressed in HeLa cells for 24 h by transfection. Whole-cell lysates were subjected to SDS-PAGE and immunoblotted with antibodies against mono- and poly-ubiquitinated proteins (P4D1). No change in the overall profile of ubiquitinated proteins was observed, indicating that *Chla*OTU reacts only on specific target(s).

Table S1. Primers used for the generation of plasmids.