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The *Chlamydia trachomatis* Tarp effector targets the Hippo pathway.

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Abstract

Chlamydia trachomatis injects bacterial effector proteins into human epithelial cells to facilitate the establishment of new infections. The chlamydial type III secreted effector translocated actin recruiting phosphoprotein (Tarp) has been shown to nucleate and bundle actin filaments. It is also believed to initiate new signaling pathways via an N-terminal phosphorylation domain. A comprehensive understanding of the host pathways that are controlled by Tarp to aid in the establishment of a successful infection remains incomplete. To gain further insight into the cell signaling regulated by Tarp, we generated transgenic fruit flies engineered to express the N-terminal domain of Tarp. As many signaling pathways are conserved between flies and mammals, we hypothesized that expression of the Tarp N-domain in the fruit fly might disrupt key pathways, resulting in developmental defects. Tarp N-domain expression in the fruit fly resulted in a mechanosensory bristle duplication phenotype similar to a previously characterized fly phenotype found to be a consequence of defects in the Hippo pathway. Tarp-dependent disruption of the Hippo pathway was confirmed in a *C. trachomatis* tissue culture infection model. The capability of Tarp to alter Hippo pathway signaling in infected epithelial cells is a previously unrecognized pathway commandeered by chlamydia and likely contributes to the establishment of chlamydia's intracellular niche.

Keywords

Chlamydia trachomatis; Tarp; Hippo pathway; YAP; LATS1/2

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Conflict of interest statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

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Introduction:

Chlamydia trachomatis is an obligate intracellular Gram-negative bacterium which has evolved to efficiently invade human cells. Distinct biovars give rise to genital, lymphatic or ocular infections [1]. Although unique tissue tropisms result in different clinical presentations, all *Chlamydia* share a common biphasic developmental cycle in which the bacteria transition between two distinct forms called the elementary body (EB) and the reticulate body (RB) [2]. Reticulate bodies are characterized as the actively growing intracellular form which replicate within the protective confines of a parasitophorous vacuole called an inclusion. Once replication is complete, the intracellular RBs differentiate into the infectious EBs and are released by simultaneous cell and inclusion lysis or by an extrusion mechanism in which the intact inclusion is ejected from the infected cell [3]. A new infection results from the cumulative efforts of multiple processes initiated once the extracellular EB attaches itself to a host cell receptor. For *C. trachomatis*, receptors such as fibroblast growth factor 2 (FGF2) and ephrin type-A receptor 2 (EphA2) have been implicated [4,5]. Receptor engagement is followed by signaling and cytoskeletal changes inside the host that drive bacterial internalization. EB entry is facilitated in part by a type III secretion system in which prepackaged effector proteins are injected into the host cell at the time of entry [6]. One effector protein called translocated actin recruiting phosphoprotein (Tarp) is responsible for driving both cytoskeletal and cell signaling changes [7-11]. Tarp is a multifunctional chlamydial protein which harbors an N-terminal phosphorylation domain and multiple C-terminal actin binding domains. The C-terminal actin binding domains directly promote actin polymerization and actin bundle formation [10,12]. The N-terminal phosphorylation domain harbors six tyrosine rich repeats and this region is capable of being phosphorylated by multiple tyrosine kinases (Src, Abl, Syk) following injection into the host cell [9,13]. Phosphorylated Tarp is known to directly associate with SH2-domain containing proteins such as phosphatidylinositol 3-kinase (PI3K) and SHC-transforming protein 1 (SHC1) [14,15]. Furthermore, phosphorylated peptides representing individual Tarp tyrosine rich repeats have been shown to associate with a complex of host cell signaling molecules including son of sevenless homolog 1 (Sos1), Abelson interactor 1 (Abi1), epidermal growth factor receptor kinase substrate 8 (Esp8) and guanine nucleotide exchange factor VAV2 (Vav2) [14]. Collectively, the recruited host cell signaling molecules are believed to direct cytoskeletal and early anti-apoptotic signals favorable for *C. trachomatis* invasion and residence in the newly infected cell. Although many Tarp interacting molecules have previously been identified with certain functions implied, it is likely that additional novel pathways have yet to be characterized. Herein we used a fruit fly (*Drosophila melanogaster*) model system to investigate Tarp-mediated signaling. As many signaling pathways are conserved between flies and mammals, we hypothesized that expression of the N-terminal domain of Tarp in the fruit fly may reveal important cell signaling perturbations through detection of characteristic developmental defects and phenotypes. Our data revealed that expression of N-terminal Tarp in the dorsal thorax of flies resulted in duplication of the large mechanosensory bristles (macrochaetes), similar to a previously described fly phenotype due to defective Hippo pathway signaling [16]. Changes in Hippo pathway signaling was also confirmed in a *C. trachomatis* tissue culture infection model in a Tarp-dependent manner. The Hippo pathway is a highly conserved kinase signaling cascade that plays a role in a

large number of cellular processes such as cell fate determination, proliferation, and survival [17]. Our findings indicate that the Tarp effector intersects the host cell Hippo pathway, which likely proves advantageous for chlamydial development.

Materials and methods:

***Drosophila melanogaster* genetics, rearing, bristle scoring and imaging.**

The open reading frame that encodes for the N-terminal region of *Chlamydia trachomatis* serovar L2 strain 434/Bu (ATCC VR-902B) Tarp (amino acids 1-431) (GenBank: [AAT47185.1](#)) was PCR amplified from *C. trachomatis* genomic DNA using primers 5'-GTTTGCGGCCGCATGACGAATTCTATATCAGGTGATC-3' and 5'-GCCCTCTAGACTACTCGTTACGAGGCCCTGTTGTG-3' and cloned into the *Drosophila* transformation and expression vector (pUAST) using the NotI and XbaI restriction sites. The sequence-verified pUAST-N-terminal Tarp plasmid was used for P-element-mediated germline transformation to generate transgenic flies [18]. The GAL4 driver stocks used are,: w[*]: *ubi-GAL4/CyO* (BDSC_32551), expresses GAL4 in all cells; y[1] w[1118]: *pnr-GAL4/TM3,UAS-y,Ser* (BSDC_3039), expresses GAL4 in tissues along the dorsal length of the fly; *hs-GAL4/CyO* (a generous gift from J. McDonald, Kansas State University) is a heat-shock-inducible ubiquitous GAL4. Viability experiments were performed at 22°C. Fly stocks and crosses were maintained at 25°C. Duplication in at least one of the dorsocentral or scutellar macrochaets was considered a bristle duplication phenotype. Fly notum (dorsal thorax) images were captured on a Zeiss Stemi 508 with an Axiocam 208 color camera.

***Chlamydia trachomatis* and host cells.**

C. trachomatis L2 (LGV 434) clones [8] were propagated in HeLa 229 cells (ATCC CCL-2.1) and purified by diatrizoate meglumine and diatrizoate sodium density gradient centrifugation [19]. HeLa 229 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% L-glutamine.

Infections and Immunoblots.

Clones of *C. trachomatis* L2 (wild type, tarp and tarp+pTarp) [8] were used to infect HeLa cells at an MOI of 100, and the cultures were incubated at 37°C for the indicated time points. Cells were harvested either using sample buffer for immunoblot analysis or washed and frozen for RNA extraction. Proteins were separated by SDS-PAGE and immunoblot performed using antibodies for the Hippo pathway (Hippo antibody sampler kit) (Cell signaling), the anti-actin C4 monoclonal antibody (Chemicon International), the anti-chlamydial Hsp60 A57-B9 monoclonal antibody (Thermo Fisher Scientific), and the anti-Tarp rabbit polyclonal antibody [7].

RNA extraction, reverse transcription, and qPCR

Host cell RNA was isolated using the RNeasy plus kit (Qiagen) and cDNA synthesized using the Quantitect reverse transcription kit (Qiagen). Quantitative PCR was performed with iQ SYBR Green Supermix (Bio-Rad) and run on a CFX96 C1000 RThermal Cycler (Bio-Rad). The primers used for amplification are: human TEAD4: forward, 5'-CTGGACAAGCCCATCGACAA-3'; reverse, 5'-AGCTTGATGTAGCGGGCAAT-3';

human XIAP: forward, 5'-TGGGGTTCAGTTTCAAGGACA-3'; reverse, 5'-CGCCTTAGCTGCTCTTCAGT-3'; and human GAPDH, forward, 5'-AGCCACATCGCTCAGACAC-3'; reverse, 5'-GCCCAATACGACCAAATCC-3'.

Statistical analysis

Statistical analyses were performed using GraphPad Prism (version 9.0.0, GraphPad) and the one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparisons test to the uninfected control.

Results

Transgenic fruit flies expressing the N-terminal domain of Tarp present with dorsal bristle duplication.

The *Chlamydia trachomatis* Tarp effector can be divided into two parts that possess unique biochemical activities (Fig. 1A). The N-terminal portion of Tarp contains 6 tyrosine-rich repeats located between amino acids 125 and 424 and is implicated in redirecting host cell signaling (Fig. 1A). In order to focus on *Drosophila melanogaster* phenotypes associated with Tarp's tyrosine-rich N-terminal domain and possible cell signaling pathways influenced by Tarp, transgenic flies were developed which expressed the Tarp N-terminal domain only. The exclusion of the C-terminal domain of Tarp was purposeful as we were interested in Tarp-mediated phenotypes unrelated to actin dynamics. Multiple fly clones containing the N-terminal Tarp transgene were successfully generated. The N-terminal Tarp transgene is under the control of the UAS promoter and depends on the presence of the GAL4 protein to drive efficient expression [20]. Crossing the UAS-N-terminal Tarp fly stocks to a ubiquitous or tissue-specific GAL4 fly stock gave rise to progeny expressing N-terminal Tarp in the tissues that expressed GAL4.

We first set out to confirm the expression of N-terminal Tarp in transgenic *Drosophila*. Ubiquitous expression of N-terminal Tarp, using *ubi*-GAL4, was found to be 100% lethal. Two independent trials using three unique transgenic stocks of N-terminal Tarp failed to generate viable *ubi*-GAL4/UAS-N-terminal Tarp adults out of 101-158 adult progeny scored per cross (Table S1). Ubiquitous expression of a control GFP transgene did not impact adult viability, producing all adult flies at the correct Mendelian ratio in both trials (Table S1). To circumvent the lethal phenotype of constitutive ubiquitous N-terminal Tarp expression, ubiquitous expression of N-terminal Tarp was transiently induced using a heat-shock-inducible GAL4. *hs*-GAL4/UAS-N-terminal Tarp adults were generated, and the flies were subjected to 2 hours of heat shock at 37°C to induce N-terminal Tarp expression. Following transgene induction, whole flies were homogenized in SDS-PAGE sample buffer. Immunoblot analysis of the whole fly lysates using anti-Tarp antibodies revealed a strong band at ~100 kDa in all *hs*-GAL4/UAS-N-terminal Tarp samples that was absent in the control *hs*-GAL4/UAS-GFP samples (Fig 1B). The N-Terminal Tarp protein size observed in the immunoblot was consistent with that of recombinant N-terminal Tarp fragments previously characterized [9].

We expressed N-terminal Tarp in a tissue-specific manner as another means to generate surviving adults. For this, we used *pnr*-GAL4, which expresses in the tissues along the dorsal midline of the adult fly [21]. This includes the dorsal portion of the fly thorax, called the notum, which contains an array of small and large mechanosensory bristles, whose pattern and number is developmentally controlled (Fig 1C, left, white dashed lines). Within this region, there are two pairs of dorsocentral macrochaetes (aDC and pDC) and 2 pair of scutellar macrochaetes (aSC and pSC). Expression of N-terminal Tarp using *pnr*-GAL4 resulted in dramatic duplication of dorsocentral and scutellar macrochaetes (Fig 1C, right, green asterisks). Bristle duplication was rare in control GFP-expressing flies (~1%) but was highly prevalent in N-terminal Tarp-expressing flies (67.5%-100%) (Table S2). Interestingly, our findings strongly phenocopied the macrochaete duplication observed upon perturbation of the Hippo pathway in the notum [16]. This suggested that Tarp alone, through its N-terminal domain, may be sufficient to impact Hippo pathway signaling.

The chlamydial Tarp effector intersects Hippo pathway signaling.

The Hippo pathway is a conserved signaling pathway first identified in *Drosophila melanogaster* but subsequently identified in mammals and is associated with many mammalian genes that function as oncogenes or tumor suppressors [17]. The bristle duplication observed in fruit flies expressing N-terminal Tarp is similar to fly phenotypes that result from mutations in the Hippo pathway, which regulates cell proliferation and apoptosis [16]. We therefore investigated whether Hippo pathway signaling was altered in chlamydia-infected cells using a panel of antibodies targeting key Hippo pathway proteins. We compared the kinetics of the Hippo pathway core signaling components in wild type *C. trachomatis* infected cells to cells infected with a mutant clone lacking the Tarp effector (*tarp*). At 6- and 8-hours post-infection, lower levels of yes-associated protein 1 (YAP), phosphorylated YAP (serine residues 127 and 397), and large tumor suppressor kinase (LATS1) were detected in the *tarp*-infected cells compared to the wild type-infected cells (Fig. 2A). In both infections YAP and WW domain-containing transcription regulator protein 1 (WWTR1 also known as TAZ) protein levels decreased between 2- and 8- hours post infection, although YAP levels decreased to a greater extent in the mutant infected cells. Interestingly, no difference in the protein levels of the mammalian homolog of the Hippo kinase, sterile-20-type kinase (STK4/3) also known as MST1 or MST 2 were found between the wild type- and *tarp*-infected cells (Fig. 2A). Because time points 4-, 6-, and 8-hours post-infection revealed Tarp-dependent alterations to YAP, phosphorylated YAP and LATS1, we expanded the study to include uninfected control cells and the Tarp complement clone for each of these time points. The results indicated that at 6- and 8-hours post-infection, YAP, phosphorylated YAP and LATS1 were reduced in the host cells infected with the Tarp mutant compared to cells infected with the wild type and complement clones. Together, these results support a role for Tarp in the modulation of the Hippo pathway during chlamydia infection.

C. trachomatis increases the mRNA levels of YAP-dependent genes in a Tarp-dependent manner.

In order to investigate potential downstream consequences of Tarp-dependent alterations in Hippo pathway signaling, the mRNA levels of YAP-dependent genes were measured. Total

RNA was isolated from uninfected and *C. trachomatis* wild type, Tarp mutant and Tarp complement infected cells after an 8-hour incubation. The levels of expression of well characterized Hippo pathway genes encoding TEAD4 [22], a transcription factor that associates with YAP to regulate gene expression and XIAP [23], a protein that inhibits programmed cell death were determined by quantitative PCR. Our results demonstrated that TEAD4 and XIAP transcripts were significantly elevated in wild type *C. trachomatis* and complement infected cells compared to the uninfected control. Interestingly, the levels of these transcripts in the tarp -infected cells were similar to or slightly lower compared to those of the uninfected cells (Fig. 3), further supporting the finding that *C. trachomatis* modulation of the Hippo pathway is Tarp-dependent.

Discussion

The tyrosine-rich repeat domain of Tarp is thought to redirect host cell signaling once delivered into the host cell. Once phosphorylated, SH2-domain containing proteins are capable of directly binding to Tarp [14,15]. The possible consequence of Tarp binding may lead to enhancement or sequestration of specific host signaling pathways. We hypothesized that additional yet undefined signaling pathways might be disrupted once Tarp is injected into a host cell. In order to explore this possibility, we leveraged a *Drosophila melanogaster* model and generated fruit flies that express the N-terminal tyrosine-rich repeat domain of Tarp. Previous studies have highlighted the utility of *Drosophila* in identifying novel functions for bacterial effectors [24]. *Drosophila melanogaster* is an established model organism with an abundance of genetic tools, reagents, and approaches available for study. More importantly, the molecular and cellular mechanisms that govern the various aspects important for *Drosophila* development are well defined. Therefore, perturbations in the proper development of easily scorable adult fly structures can often be traced back to the relevant molecular pathways. Using transgenic expression, *Drosophila* becomes a powerful *in vivo* platform to investigate the impact of the *C. trachomatis* Tarp effector on host cell biology without the confounding influence of active infection. Herein, we focused on elucidation of molecular events specifically associated with the N-terminal portion of Tarp. Interestingly, N-terminal Tarp expression in the tissues that give rise to the dorsal thorax, or notum, of flies produced a striking and highly penetrant duplication of the large mechanosensory bristles. This phenotype has been described previously for flies in which Hippo pathway signaling has been disrupted [16]. The Hippo pathway was first characterized in *Drosophila* and is associated with organ development [25]. Subsequent studies revealed that the Hippo pathway is conserved in mammals albeit with additional levels of homeostatic regulation [26]. In order to verify that *Chlamydia trachomatis* infection affects the Hippo pathway of host cells in a Tarp-dependent manner, mammalian Hippo pathway homologs were assessed by immunoblot analysis of uninfected and *C. trachomatis* infected cells. Interestingly, YAP (the mammalian homolog of *Drosophila*'s Yorkie transcriptional coactivator), phosphorylated YAP and LATS1 (the mammalian homolog to *Drosophila*'s Warts kinase), were found to be reduced in tarp -infected cells compared to wild type and complement controls. Unphosphorylated YAP is the active form of YAP, which can promote expression of transcriptional targets that regulate cell growth, proliferation and apoptosis (anti-apoptotic factors). Our data indicate that the absence of

Tarp results in lower overall amounts of YAP (and TAZ) and LATS1 in infected host cells, suggesting that *C. trachomatis* infection intersection of the Hippo pathway is Tarp-dependent. Furthermore, expression of YAP/TAZ-TEAD regulated target genes, XIAP and the co-transcription factor TEA-domain family member (TEAD) itself [22] was significantly increased in chlamydia infected cells compared to uninfected control cells. Induction of XIAP and TEAD transcripts by chlamydia infection required Tarp. Although the reduced signal for phosphorylated YAP in the Tarp mutant might imply the potential for greater expression of target genes, we believe the reduced phosphorylation level of YAP is likely a consequence of reduced total levels of YAP in the Tarp mutant infected cells. Therefore, we propose that the Tarp effector slows down YAP and LATS1 degradation by an unknown mechanism, leading to expression of YAP/TAZ-TEAD target genes.

Other pathogens have developed ways to hijack the Hippo pathway. Infections of human umbilical vein endothelial cells (HUVEC) by the intracellular apicomplexan parasite *Toxoplasma gondii* results in increased phosphorylation of LATS1 and cytosolic retention of YAP leading to reduced YAP target gene expression [27]. *Helicobacter pylori*, a bacterium associated with gastric and duodenal ulcers and gastric cancer delivers CagA to gastric epithelial cells via a type IV secretion system (T4SS). A recent study revealed that deletion of *cagA* results in reduced expression of YAP in *H. pylori* infections [28]. Furthermore, lower levels of YAP in the absence of *cagA* suppressed *H. pylori* induced epithelial-mesenchymal transition (EMT) drawing a mechanistic link between *H. pylori* infections and gastric cancer [28]. The intracellular pathogen *Legionella pneumophila* employs the effector LegK7 to hijack the Hippo signaling pathway in mammalian macrophages [29]. LegK7 acts as a Hippo/MST1 kinase imposter to phosphorylate the scaffolding protein Mps one binder kinase activator 1A (MOB1A). MOB1A phosphorylation consequentially leads to the degradation of TAZ and YAP and a transcriptional profile more advantageous for bacterial intracellular growth and survival [29].

Whether the chlamydial Tarp effector can disrupt YAP and TAZ through one or more of the mechanisms used by other pathogens is unknown. The precise interplay between the mammalian homologs in the pathway that lead to reduced levels of YAP and TAZ remain to be determined but is a promising avenue for future research. Collectively, our work provides evidence that the *C. trachomatis* effector Tarp is able to alter the Hippo pathway in infected host cells and this potentially contributes to the mechanisms that chlamydia uses to hijack host cell functions to promote infection and pathogenesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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- The N-domain of Tarp expressed in the fruit fly results in bristle duplications.
- The chlamydial effector Tarp intersects the Hippo signaling pathway.
- *Chlamydia trachomatis* infected cells demonstrate altered Hippo signaling

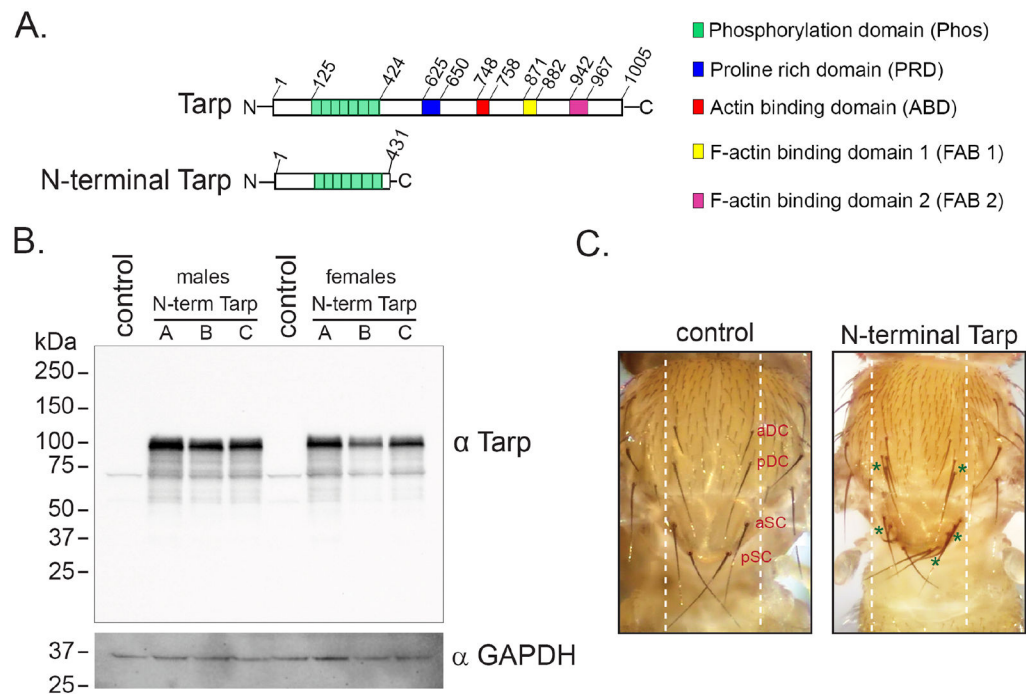


Figure 1. The N-terminal fragment of *C. trachomatis* Tarp induces mechanosensory bristle duplication in *Drosophila melanogaster*.

(A) Schematic diagram of full-length Tarp (top) and the N-terminal fragment (amino acids 1-431) of Tarp (bottom). The functional domains of Tarp are indicated. (B) Expression of the N-terminal Tarp protein in transgenic flies is confirmed by immunoblot. Immunoblot analysis using anti-Tarp antibodies (α Tarp) was performed on whole fly lysates following a 2-hour 37°C heat shock of hs-GAL4/UAS-N-terminal Tarp fly lines (N-term Tarp A, B and C) and hs-GAL4/UAS-mCD8:GFP (control) flies. GAPDH was used as a loading control (α GAPDH). (C) Representative images of the *Drosophila* notum of *pnr*-GAL4/UAS-mCD8:GFP (control) flies and *pnr*-GAL4/UAS-N-terminal Tarp (N-terminal Tarp) flies. The normal pattern and number of dorsocentral bristles, anterior (aDC) and posterior (pDC), as well as scutellar bristles, anterior (aSC) and posterior (pDC) is shown in the control. Transgene expression occurs between the white dashed lines. Excess dorsocentral and scutellar bristles in the N-terminal Tarp image are indicated with green asterisks.

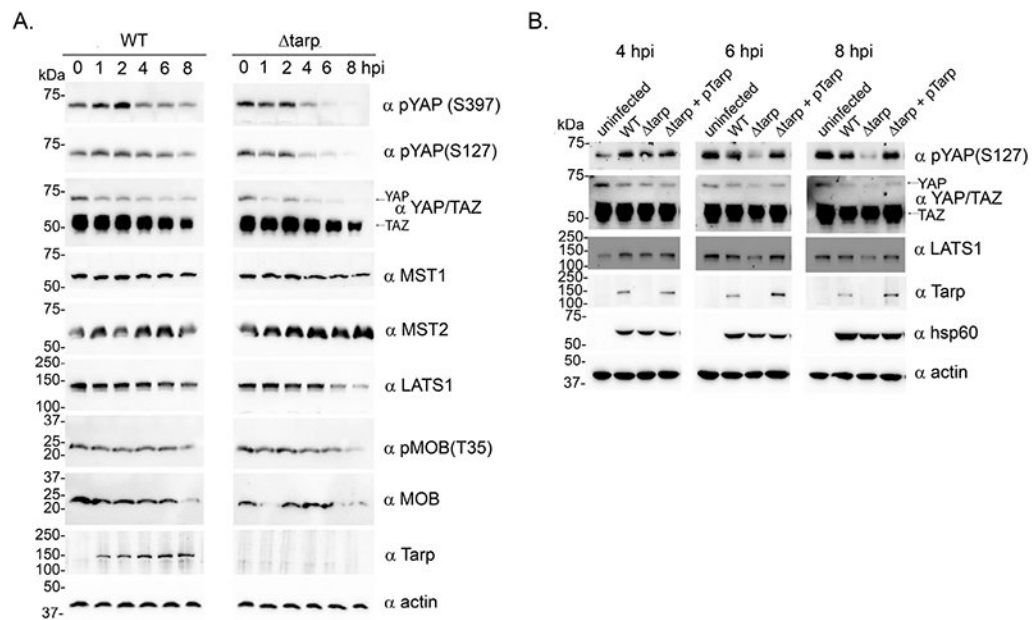


Figure 2. *C. trachomatis* affects the Hippo pathway in a Tarp-dependent manner.

(A) HeLa cells were infected with either wild-type *C. trachomatis* L2 (WT), or a *tarP* deletion mutant ($\Delta tarp$) for 0-, 1-, 2-, 4-, 6- or 8-hours. Culture material was harvested, and protein lysates were generated. Proteins were resolved by SDS-PAGE and immunoblots were performed with actin (α actin), Tarp (α Tarp), large tumor suppressor (α LATS), Yes-associated protein (YAP) and WW domain-containing transcription regulator protein1 (WWTR1 also known as TAZ) are both recognized by the same polyclonal sera (α YAP/TAZ), mammalian sterile-20-like 1 (α MST1), mammalian sterile-20-like 2 (α MST2), phosphorylated YAP (α pYAP^{S397} and α pYAP^{S127}), mps one binder kinase activator-like 1A and 1B (α MOB) and phosphorylated MOB (α pMOB^{T35}) (B) Similar to (A) with the addition of uninfected control cells (uninfected) and HeLa cells infected with *C. trachomatis* mutant complemented with pTarp ($\Delta tarp$ + pTarp) at 4-, 6- and 8- hours. Antisera specific for *C. trachomatis* heat shock protein 60 (α hsp60) served as an additional control.

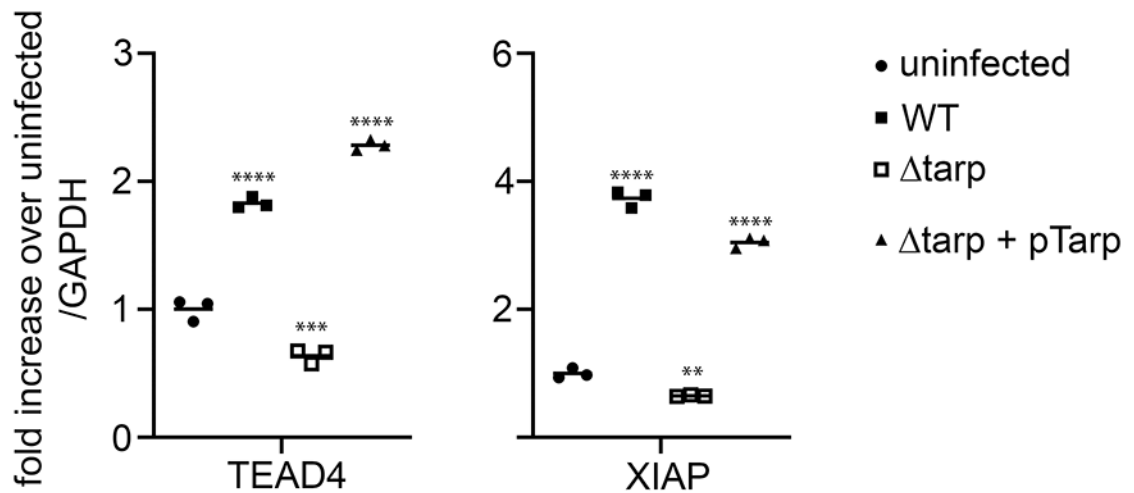


Figure 3. YAP regulated transcripts TEAD4 and XIAP are induced during *C. trachomatis* infection in a Tarp-dependent manner.

HeLa cells were infected with *C. trachomatis* L2 (WT), a *tarP* deletion mutant (Δ tarP), or the mutant complemented with pTarP (Δ tarP + pTarP). Mock infected cells (uninfected) served as the negative control. RNA was harvested from uninfected or infected cells 8 hours post infection. Data are presented as the relative fold change in transcriptional enhancer factor TEF 4 (TEAD4) and X-linked inhibitor of apoptosis protein (XIAP) mRNA levels in infected versus uninfected cells normalized to GAPDH transcripts. Statistical analysis was performed using a one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparisons test to the uninfected control (** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).