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The AAA+ ATPase ClpX Is Critical for Growth and Development of *Chlamydia trachomatis*

Nicholas A. Wood[1], Amanda M. Blocker[2], Mohamed A. Seleem[3], Martin Conda-Sheridan[3], Derek J. Fisher[2], Scot P. Ouellette[1#]

[1]Department of Pathology and Microbiology, College of Medicine, University of Nebraska Medical Center, Omaha, NE

[2]School of Biological Sciences, Southern Illinois University Carbondale, Carbondale, IL

[3]Department of Pharmaceutical Sciences, College of Pharmacy, University of Nebraska Medical Center, Omaha, NE

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#Corresponding Author:

Department of Pathology and Microbiology, College of Medicine, University of Nebraska Medical Center, 985900 Nebraska Medical Center (DRC2 5022), Omaha, NE

Tel: +1-402-559-0763 Fax: +1-402-559-5900 Email: scot.ouellette@unmc.edu

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Abstract:

Chlamydia trachomatis (Ctr) is an obligate intracellular bacterium that undergoes a complex developmental cycle in which the bacterium differentiates between two functionally and morphologically distinct forms, each of which expresses its own specialized repertoire of proteins. The transitions between the infectious, non-dividing elementary body (EB) and the non-infectious, replicative reticulate body (RB) are not mediated by division events that re-distribute intracellular proteins. Rather, both primary (EB to RB) and secondary (RB to EB) differentiation require protein turnover. The Clp protease system is well conserved in bacteria and, minimally, relies on a serine protease subunit, ClpP, and a AAA+ ATPase, such as ClpX, that recognizes and unfolds substrates for ClpP degradation. In *Chlamydia*, *clpX* is encoded within an operon adjacent to *clpP2*. We present evidence that the chlamydial ClpX ortholog, and the co-transcribed ClpP2, play a key role in organism viability and development. We demonstrate here that chlamydial ClpX is a functional ATPase and forms the expected homohexamer *in vitro*. Overexpression of a ClpX mutant lacking ATPase activity had a limited impact on DNA replication or secondary differentiation but, nonetheless, reduced EB viability. Conversely, the overexpression of an inactive ClpP2 mutant significantly impacted later developmental cycle progression by reducing the overall number of organisms. Blocking *clpP2X* transcription using CRISPR interference led to a decrease in bacterial growth, which did not occur when the non-essential gene *incA* was targeted. Taken together, our data indicate that ClpX and the associated ClpP2 play a critical role in developmental cycle progression and differentiation.

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Importance

Chlamydia trachomatis is the leading cause of infectious blindness globally and the most reported bacterial sexually transmitted infection both domestically and internationally. Given the economic burden, the lack of an approved vaccine, and the use of broad-spectrum antibiotics for treatment of infections, a further understanding of chlamydial growth and development is critical for the advancement of novel, targeted antibiotics. The Clp proteins comprise an important and conserved protease system in bacteria. Our work highlights the importance of the chlamydial Clp proteins to this clinically important bacterium. Additionally, our study implicates the Clp system playing an integral role in chlamydial developmental cycle progression, which may help establish models of how *Chlamydia* spp. and other bacteria progress through their respective developmental cycles. Our work also contributes to a growing body of Clp-specific research that underscores the importance and versatility of this system throughout bacterial evolution and further validates Clp-proteins as drug targets.

Words: 150/150

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Introduction

Chlamydia trachomatis (Ctr) is the leading cause of both bacterial sexually transmitted infections (STIs) and infectious blindness worldwide (1, 2). When left untreated, STIs can result in chronic sequelae, including pelvic inflammatory disease, ectopic pregnancy, and tubal infertility. A better understanding of Ctr molecular processes may help reveal essential systems that can be leveraged for more targeted intervention strategies.

Chlamydia species are obligate intracellular bacterial pathogens that differentiate between distinct functional and morphological forms during the course of their developmental cycles (see (3) for a comprehensive review). The elementary body (EB) is small (~0.3 μm in diameter), infectious, but non-dividing (4, 5). An EB attaches to a host cell and is internalized into a host membrane-derived vacuole that is rapidly modified into the inclusion (6-9). Within this inclusion, the EB undergoes primary differentiation into the larger (~1.0 μm in diameter) reticulate body (RB). The RB is non-infectious but divides using a polarized budding mechanism (10) until secondary differentiation from an RB to an EB occurs. Studies over the years have extensively detailed the transcriptional and proteomic differences between EBs and RBs (e.g. (11-14)). Given that chlamydial differentiation is not preceded by an unequal division and redistribution of intracellular proteins, as occurs in other bacteria such as *Bacillus subtilis* (see (15) for review) or *Caulobacter crescentus* (16), and that EBs and RBs have distinct proteomes, we hypothesize that proteomic turnover plays an integral role in chlamydial differentiation.

Previously, our groups characterized the two ClpP paralogs of Ctr. We established that the core *clp* protease-associated genes are expressed in the mid-developmental cycle and that ClpP1 and ClpP2 likely perform unique roles in chlamydial physiology (17). In addition to the two ClpP paralogs, *C. trachomatis* encodes a ClpX homolog (18). ClpX is a Type I AAA+

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(**A**TPase **A**ssociated with diverse cellular **A**ctivities) unfoldase that utilizes ATP hydrolysis to linearize protein substrates for either degradation by the ClpP protease or refolding (19, 20). Type I AAA+ ATPases encode Walker A and Walker B motifs, which are responsible for ATP binding and hydrolysis, respectively (21, 22). ClpX oligomerizes to form a homo-hexamer that then recognizes substrates through multiple different mechanisms (see (23) for a comprehensive review).

Here, we characterized the role of ClpX in chlamydial growth and development. Because *clpX* is encoded within an operon with *clpP2*, we investigated effects of overexpression and knockdown of both components. Ctr ClpX is highly conserved, possesses ATPase activity, and formed the expected homohexamer *in vitro*. Interestingly, overexpression of wild-type ClpX, ClpP2, and ClpP2X constructs in Ctr had little effect on bacterial growth, but overexpression of the inactive mutants (alone or in tandem) negatively impacted recoverable inclusion forming units (IFUs). However, the reduction in IFUs upon inactive ClpX overexpression resulted from non-functional EB generation while the IFU reduction upon inactive ClpP2 overexpression was the result of a block in developmental cycle progression. Our results indicate that chlamydial ClpX is a true ortholog of bacterial ClpX and that the ClpP2X system is integral to chlamydial growth and development.

Words: **498/500**

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Results

The chlamydial ClpX retains conserved motifs of, and exhibits predicted structural homology to, ClpX orthologs. To initiate our study, we first performed bioinformatic and *ab initio* structural modeling analyses to determine whether the chlamydial ClpX ($ClpX_{Ctr}$) possesses the expected conserved regions and motifs consistent with its proposed function as an AAA+ ATPase. Using multiple sequence alignment, we aligned $ClpX_{Ctr}$ to ClpX orthologs and annotated conserved motifs identified in other studies (Fig. 1a). $ClpX_{Ctr}$ retains the N-terminal metal binding domain (24, 25), the Walker A and B motifs for ATP binding and hydrolysis, respectively (21, 23), the sensor motifs for recognition of nucleotide bound state (26), the RKH motif and pore loops for substrate recognition (27-29) and unfolding (30, 31), the arginine finger for inter-subunit sensing of nucleotide state in the ClpX hexamer (22, 32), and the IGF Loop for interaction with ClpP (33, 34). Interestingly, the predicted secondary structure of $ClpX_{Ctr}$ shows few notable aberrations (see Discussion) from other prototypical bacterial ClpX orthologs and is predicted to form the expected homohexamer by structural modeling (Fig. 1b, two subunits removed for clarity). The spatial conservation of AAA+ and ClpX-specific motifs (colored in Fig. 1b as in the multiple sequence alignment) indicates that the chlamydial ClpX likely functions using a mechanism similar or identical to other ClpX orthologs. Taken together, these *in silico* studies suggest $ClpX_{Ctr}$ functions as a canonical AAA+ ATPase.

Chlamydial ClpX forms the expected homohexamer and possesses ATPase activity. To determine the oligomeric state of $ClpX_{Ctr}$ *in vitro*, we purified recombinant protein and analyzed its migration by native PAGE. At the same time, we also constructed a Walker B ATPase mutant (E187A) $ClpX_{Ctr}$ as a control for biochemical studies. Following the incubation of 10 μg of wild-

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type or mutant $ClpX_{Ctr}$ for 20 minutes in a HEPES based buffer, we loaded the entire volume into a 4-20% gradient gel. We observed the $ClpX_{Ctr}$ proteins migrating above the 242 kDa band of the molecular weight ladder, which is close to the expected hexameric size of 283 kDa (Fig. 2a). We then sought to assess ATPase activity of recombinant wild-type and ATPase mutant $ClpX_{Ctr}$ using the Biomol Green endpoint assay to measure free phosphate levels following ATP hydrolysis, which served as a proxy for ATPase activity. Indeed, $ClpX_{Ctr}$ hydrolyzed ATP, while the inactive mutant isoform showed a significant defect in ATP hydrolysis (Fig. 2b). These data indicate that $ClpX_{Ctr}$ (i) forms a homohexamer of the predicted size and (ii) possesses ATPase activity that is abrogated by a mutation in the Walker B motif.

We next tested whether wild-type and ATPase mutant $ClpX_{Ctr}$ interact with each other using the Bacterial Adenylate Cyclase Two-Hybrid (BACTH) assay. This system is predicated on the reconstitution of adenylate cyclase activity by bringing two complementary fragments of the enzyme (T25 and T18) into close proximity by interacting proteins. Generation of cAMP by the reconstituted adenylate cyclase drives ß-galactosidase production that can be measured qualitatively by the presence of blue colonies and growth on minimal medium (Fig. 2c) or quantitatively by measuring enzyme activity directly (Fig. 2d). We performed a series of pairwise interaction tests between the wild-type and mutant $ClpX_{Ctr}$. In each instance, we observed a positive interaction that was quantifiable and on par with the positive control (T25-Zip vs T18-Zip). We conclude from these data that the mutant isoform can interact with the wild-type isoform.

Overexpression of inactive ClpX or inactive ClpP2 has both overlapping and independent effects. We previously measured the effects of overexpression of both wild-type and catalytically

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150 inactive $ClpP2_{Ctr}$ on chlamydial growth and observed a modest reduction in growth at 24 hours
151 post-infection (hpi) (17). We wanted to more carefully assess growth differences during the
152 chlamydial developmental cycle in the presence of overexpressed wild-type and mutant $ClpX_{Ctr}$
153 (and $ClpX_{E187A}$), $ClpP2_{Ctr}$ (and $ClpP2_{S98A}$), or both together ($ClpP2X_{Ctr}$/$ClpP2_{S98A}ClpX_{E187A}$). To
154 do this, we performed growth curves where we induced expression, or not, at 10 hpi and
155 quantified growth at various timepoints after induction. Immunofluorescence analysis (IFA) of
156 replicate treatments and quantification of recoverable inclusion forming units (IFUs; a proxy for
157 EBs) revealed distinct effects upon overexpression of the individual components (Fig. 3a-c) as
158 well as with the entire operon (Fig. 3d&e). We noted that overexpression of wild-type $ClpP2_{Ctr}$
159 showed no appreciable effect at either 24 or 48 hpi (14 and 38 h pulses of induction,
160 respectively), whereas overexpression of $ClpP2_{S98A}$ appeared to reduce the number of organisms
161 present within the inclusion at 48 hpi but not 24 hpi (Fig. 3a). These observations correlated with
162 the marked impact on EB production in the later time points of mutant $ClpP2_{S98A}$ but not wild-
163 type $ClpP2_{Ctr}$ overexpression (Fig. 3b). Conversely, inactive $ClpX_{E187A}$ overexpression resulted
164 in smaller inclusions and a decrease in IFUs that was not observed for overexpression of the
165 wild-type ClpX (Fig. 3a&c). These IFU recovery data suggest that Ctr is more sensitive to
166 $ClpX_{Ctr}$ rather than $ClpP2_{Ctr}$ disruption earlier in the developmental cycle, as the IFU reduction is
167 exacerbated sooner with $ClpX_{E187A}$ overexpression (note the differences at 24hpi in Fig. 3b&c).
168 As noted for the overexpression of individual wild-type isoforms, there was no significant impact
169 on IFU recovery of overexpressing both wild-type $ClpP2_{Ctr}$ and $ClpX_{Ctr}$ in tandem. Consistent
170 with the effects of overexpressing individual mutant isoforms, overexpression of the inactive
171 $ClpP2_{S98A}$ and $ClpX_{E187A}$ isoforms in tandem showed an exacerbated phenotype throughout the
172 developmental cycle as noted by both IFA and IFU assays (Fig. 3d&e). Importantly, the wild-

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type chromosomal copies of $ClpP2_{Ctr}$ and $ClpX_{Ctr}$ continue to be expressed during these overexpression assays. Therefore, the true impact of overexpression of the mutant isoforms is likely underrepresented.

Functional disruption of ClpP2 blocks developmental cycle progression while ClpX disruption reduces EB viability. Given that the IFU assay only measures EB viability from a population and not total bacterial numbers or differentiation status, we next wanted to address these nuances of the chlamydial developmental cycle. We first measured genomic DNA as a proxy for total number of bacteria (i.e. both RBs and EBs). From 24 hpi to 48 hpi, we observed a significant drop in gDNA levels when $ClpP2_{S98A}$ was overexpressed alone or in the mutant operon configuration (Fig. 4a). Conversely, overexpression of any wild-type protein had no significant impact on DNA accumulation. Surprisingly, overexpression of the $ClpX_{E187A}$ also had no significant impact on DNA levels in spite of the reduction in IFUs, suggesting total bacterial numbers are unaffected. To determine differentiation status, we next assessed HctB levels, an EB-specific gene product (35-37), by western blot as an indicator of secondary differentiation. We normalized the integrated density of HctB to the integrated density of MOMP (major outer membrane protein; present in both EBs and RBs) to ensure that we were comparing HctB levels to the total number of bacteria. The relative HctB levels in samples where $ClpP2_{S98A}$ was overexpressed were reduced substantially, suggesting the generation of fewer EBs and consistent with IFU and genomic DNA data, whereas the other experimental conditions showed no changes in relative HctB levels (Fig. 4b&c). These data suggest that overexpression of $ClpX_{E187A}$ does not impact bacterial replication, as measured by gDNA levels, or RB-to-EB differentiation, as measured by HctB levels. Therefore, we prepared samples for transmission electron microscopy

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to examine at higher resolution the morphology of EBs and RBs from $ClpP2_{S98A}$ and $ClpX_{E187A}$ overexpressing strains. Consistent with other measured effects, $ClpP2_{S98A}$ overexpression resulted in smaller inclusions with fewer organisms (Suppl. Fig. 1a). In contrast, but consistent with its measured effects, $ClpX_{E187A}$ overexpression did not have an obvious effect on RB size or numbers *per se*; rather, more bacteria with unusual, multi-nucleate staining were observed, as indicated by the arrows (Suppl. Fig. 1b&d compared to uninduced in panel C). These abnormal forms may potentially be EBs with defects in chromosomal packaging or intermediate bodies that have not completed chromosomal condensation. Taken together with the IFU data (Fig. 3), these results suggest differential effects of overexpression of $ClpP2_{S98A}$ and $ClpX_{E187A}$ and, by inference, differential effects of these Clp components in the physiology of the organism.

Knockdown of the* clpP2X *operon reduces recoverable progeny and results in reduced plasmid retention. Overexpression of mutant isoforms of $ClpP2_{Ctr}$ and/or $ClpX_{Ctr}$ was sufficient to disrupt chlamydial development in the presence of endogenous $ClpP2X_{Ctr}$. However, we wanted to directly block the chromosomal copies by employing an improved version of the chlamydial CRISPR interference (CRISPRi) strategy previously described by us ((38) and Ouellette, *in prep*). CRISPRi relies on the inducible expression of a catalytically inactive Cas9 (dCas9) in combination with a guide RNA (gRNA) to block transcription at specific chromosomal sites (39). We transformed Ctr L2 with vectors encoding the dCas9 and gRNAs targeting either the *clpP2X* or *incA* intergenic regions. IncA knockdown served as a control since *incA* is a non-essential gene (40). The CRISPRi transformants were used to infect HEp2 cells. When dCas9 expression was induced at 10hpi, we observed a marked and rapid decrease in both *clpP2* and *clpX* transcript levels compared to the uninduced controls at 14hpi (Fig. 5a). Similar results were

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observed when dCas9 expression was induced at 4hpi (data not shown). Importantly, we did not observe a decrease in transcript levels for *clpP1, euo,* and *omcB* (Suppl. Fig. 2; (12, 17, 41, 42)). As previously observed, IncA expression was uniformly blocked after dCas9 induction (Fig. 5b; (38)).

We next assayed chlamydial growth as measured by IFU recovery after inducible knockdown of the target genes. Expression of dCas9 was induced at 4hpi, and IFUs were harvested at 24 and 48hpi and titred on fresh cell monolayers in the presence of penicillin, the selection agent. When *clpP2X* expression was blocked at 4hpi, we noted a 5-fold decrease in penicillin-resistant (i.e. transformants containing the CRISPRi plasmid) IFUs at 24hpi but a more than 200-fold decrease at 48hpi (Fig. 5c). In performing these assays in the presence of penicillin, we observed numerous penicillin-sensitive organisms (i.e. aberrant RBs (43)) during the titration step, suggesting that the plasmid conferring resistance and encoding the CRISPRi system was being lost after induction of dCas9 expression. To test this, we quantified plasmid retention in the *clpP2X* knocked down samples and observed that blocking *clpP2X* expression resulted in ~75% plasmid loss at 24hpi and greater than 90% loss at 48hpi (Fig. 5d). These effects on IFUs and plasmid retention were not observed for *incA* knockdown (Fig. 5c and d). We note that *incA* knockdown did result in a reproducible, but transient, increase in IFUs at 24hpi that returned to "normal" levels at 48hpi (Fig. 5c). The reasons for this are not clear. Nonetheless, we conclude from these data that blocking *clpP2X* expression is deleterious to *Chlamydia*, further highlighting its essentiality to this pathogen.

Chemical disruption of ClpX function is detrimental to Ctr. Recently, ClpX-specific inhibitors were synthesized by the Sieber group and shown to interfere with ClpX ATPase activity. One

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compound, identified as 334, was shown to have potent inhibitory activity towards ClpX whereas a derivative, 365, was inactive (44). We performed *ab initio* modelling and molecular dynamics simulations (45) to determine if these compounds could interact with an ADP-bound hexameric $ClpX_{Ctr}$. For 334, a high scoring model (-9.1 kcal/mol binding affinity, RMSD ~ 0) was predicted with the drug binding near to the ATP binding pocket, suggesting a mechanism of action where 334 likely occludes the ATPase site (Suppl. Fig. 3). Whether the effect stems from the blocking of ATP binding and subsequent destabilization of the complex, attenuation of ATPase function by preventing a conformational change of the complex, or steric hindrance of complex formation remains to be elucidated. Conversely, compound 365 bound outside of the ATP pocket with a much lower score (Suppl. Fig. 4).

Given the predicted effects of the ClpX inhibitors on the structure of $ClpX_{Ctr}$, we next leveraged these compounds to assess the effect of specifically disrupting $ClpX_{Ctr}$ on chlamydial growth. We initiated our studies by treating or not *C. trachomatis* L2 infected HEp2 cells at 8 hpi with 25 μg of drug to target specifically RBs early in development. At 24 hpi, we either harvested and froze IFUs or replaced the medium containing either the drug or the vehicle control with fresh medium lacking these. The latter samples were harvested at 48 hpi and frozen, and then all collected samples were titred in the absence of drug treatment. Initial assessment of immunofluorescent controls showed a marked reduction in inclusion size after 334 treatment for both the 24 and 48 h timepoints (8-24h and 8-48h; Fig. 6a&b). This was accompanied by a severe decrease in recovery of IFUs to near the limit of detection (Fig. 6c). As expected, 365 treatment had little effect on IFU recovery at 24 hpi but did reduce IFU numbers by a log following prolonged treatment (8-24h and 8-48h; Fig. 6a-c), supporting our docking simulation that showed lower affinity of 365 to $ClpX_{Ctr}$. Moreover, 334 had a bacteriostatic effect on *C.*

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trachomatis, as removal at 24 hpi allowed for a substantial recovery in IFU counts (8-24h; Fig. 6b&c).

We then sought to assess the importance of $ClpX_{Ctr}$ function throughout the developmental cycle by treating either early, to target primary differentiation and inclusion establishment, or later, to target pre-formed EBs. Treatment with 334 from 0 to 8 hpi resulted in over a log reduction in recoverable IFUs, demonstrating the importance of $ClpX_{Ctr}$ early during the developmental cycle (0-8h; Fig. 6a&c). Addition of 334 following 24 h of no treatment confirmed the bacteriostatic nature of the drug's effect on *C. trachomatis*, as the IFU titre failed to increase more than a log over the 24 h untreated samples (24-48h; Fig. 6b&c). Furthermore, the addition of 334 at 24 hpi suggests that preformed EB viability is not reduced following $ClpX_{Ctr}$ inhibition. Whether this is due to the lack of drug permeability into the EBs or the reversibility of drug binding upon removal from the media is not clear. Overall, these data highlight the importance of $ClpX_{Ctr}$ for chlamydial development.

Discussion

Given the unique roles and protein repertoires of the chlamydial developmental forms (EB/RB), we hypothesize that protein degradation is a critical factor in the differentiation process from one form to the other. The Clp system is highly conserved in both prokaryotic and eukaryotic systems where it has been described to perform important functions in both proteostasis and pathogenesis (46). The Clp system is nominally composed of a proteolytic subunit, ClpP, and a AAA+ ATPase that functions as an unfoldase to recognize substrates and feed them into the ClpP barrel for degradation (23). The work presented here expands our understanding of the chlamydial Clp protease system. Focusing on an initial characterization of

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288 $ClpX_{Ctr}$ and the role of the *clpP2X* operon, we demonstrated the importance of the Clp protease
289 system during chlamydial growth and development.

290 Multiple lines of evidence support that the chlamydial ClpX is a *bona fide* AAA+
291 ATPase. Firstly, multiple sequence alignment of $ClpX_{Ctr}$ to orthologs of other bacteria revealed a
292 perfect conservation of the motifs involved in nucleotide binding, ATP hydrolysis, and
293 nucleotide-state sensing (Fig. 1) (47, 48). Secondly, homology-directed and *ab initio* modelling
294 of $ClpX_{Ctr}$ revealed that the spatial orientation of these domains is conserved as well (Fig. 1),
295 though we acknowledge that structural studies are critical to drawing conclusions about $ClpX_{Ctr}$
296 conformational states. Thirdly, $ClpX_{Ctr}$ interacts with itself to form a homohexamer that
297 possesses ATPase activity (Fig. 2). Importantly, this ATPase activity could be disrupted by a
298 targeted mutation in the Walker B motif while having no effect on the oligomerization properties
299 of the protein. Fourthly, a characterized ClpX inhibitor that disrupts its ATPase activity also
300 disrupted the growth of *C. trachomatis* serovar L2 (Fig. 6). Finally, overexpression of a $ClpX_{Ctr}$
301 ATPase mutant negatively impacted chlamydial growth and development (Figs. 3&4).

302 While we have characterized the ATPase function of $ClpX_{Ctr}$ and its role in chlamydial
303 growth, further work remains to determine whether this ClpX ortholog functions as an unfoldase.
304 Nevertheless, our bioinformatics analysis supports this as $ClpX_{Ctr}$ retains substrate recognition
305 motifs, including both pore loops and the RKH motif for gripping and translocation of substrates
306 (27-31). *Chlamydia* spp. also encode the tmRNA/ssrA tagging system for ribosomal rescue (18,
307 49-52), which fits a model where $ClpX_{Ctr}$ may play an integral role in turnover of tagged,
308 partially translated peptides. Whether $ClpX_{Ctr}$ can actually target SsrA-tagged substrates, and
309 whether this tagging is for ribosomal rescue or more specific purposes (53, 54), remains to be

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determined and is currently under investigation by our research group. A recent article, using an SsrA-tagged GFP, suggests this function of chlamydial ClpX may be conserved (55).

One unique feature of $ClpX_{Ctr}$ is the TSSTSSP link between the zinc binding domain (ZBD) and the rest of the protein. To date, the structure of the ZBD has not been crystallized with the rest of the protein due to its apparent disorder; yet, the ZBD of ClpX is important for its function in other bacteria to, for example, recognize specific substrates (24, 56, 57). We hypothesize that the TSSTSSP residues may serve a function in flexibility (58, 59) or extension of the N-terminus, which in turn may modulate its unfoldase/chaperone activity (60). Interestingly, an SP motif has been implicated in initiation of a Type I β-hairpin turn (61, 62), which may serve as a mechanism through which the $ClpX_{Ctr}$ N-terminus adopts a unique conformation to recognize uncharacterized adaptors. This linker may be phosphorylated, leading to a conformational switch of the intrinsically disordered N-terminus and enhancing the stability of the otherwise disordered ZBD. We are investigating the potential for a phosphorylation state to activate or attenuate $ClpX_{Ctr}$ function. We hypothesize that at least one of these situations aids in selectivity of $ClpX_{Ctr}$ *in vivo* activity, but we cannot rule out that any combination may function to yield multiple layers of control.

In *Chlamydia*, *clpX* is encoded in an operon with *clpP2*. Our data indicate that, not surprisingly, the $ClpP2X_{Ctr}$ system is highly regulated and essential. We previously demonstrated that unregulated $ClpP_{Ctr}$ activity, through the use of ClpP-activating antibiotics, is detrimental to *Chlamydia* (17). Here, we performed a systematic analysis of the effects of overexpression of wild-type or inactivated $ClpP2X_{Ctr}$ components. The overexpression of wild-type $ClpP2_{Ctr}$ and/or $ClpX_{Ctr}$ had no biologically or statistically significant effect on chlamydial growth that we could measure. However, overexpressing inactive $ClpP2_{Ctr}$(S98A) and/or $ClpX_{Ctr}$(E187A) resulted in

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abrogation of chlamydial growth as measured by recovery of infectious progeny. Three observations should be noted. Firstly, the effect of inducibly-expressed proteins is measured in the presence of the endogenous chromosomally-expressed proteins. Therefore, it is likely that the inactive mutants would have even more dramatic effects on chlamydial growth in the absence of the wild-type chromosomal copy. For $ClpX_{Ctr}$, this is supported by the effects of the ClpX inhibitor on *Chlamydia* (Fig. 6), which effectively stopped chlamydial growth. Secondly, we demonstrated that the mutant proteins could interact *in vitro* with wild-type isoforms (Fig. 2). Therefore, we can infer that overexpression of the mutant proteins leads to their incorporation into the endogenous ClpX machinery to disrupt or impair its function. Thirdly, to our knowledge, ours is the first study to ectopically express two different tagged proteins in *Chlamydia*, showing both the feasibility of this approach and its potential utility to dissect chlamydial biology.

The overexpression of the catalytically inactive mutant Clp proteins in *Chlamydia* revealed potentially subtle differences in the role of each component in chlamydial growth and development. Surprisingly, we noted a roughly 50% reduction in detectable genomes (Fig. 4A) when $ClpX_{Ctr}$(E187A) was expressed whereas IFUs were reduced roughly 20-fold (Fig. 3). The production of EBs as measured by HctB levels did not appreciably change (Fig. 4b&c). This suggests that, while development is hindered, the drop in IFUs may be due to defective EB viability, infectivity, or inclusion establishment and not a defect in secondary differentiation *per se*. Support for this comes from electron microscopy images, which revealed unusual morphologies after overexpression of the mutant $ClpX_{Ctr}$ isoform (Suppl. Fig. 1). Conversely, for $ClpP2_{Ctr}$(S98A) overexpression, the substantial IFU decrease coupled with a sharp drop in gDNA levels indicate that $ClpP2_{Ctr}$ plays a role in developmental cycle progression. HctB levels are also significantly reduced, which is consistent with the lack of EB generation. Taken together, these

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data may indicate that $ClpP2_{Ctr}$ is integral to developmental cycle progression or differentiation and that its function is tightly regulated. We cannot, however, exclude that secondary differentiation is directly affected due to the fact that total organism numbers are severely reduced. Rather, our proposed model suggests that $ClpP2_{Ctr}$ disruption may affect both factors by a mechanism that we are currently working to identify. Conversely, $ClpX_{Ctr}$ may serve a more prominent $ClpP2_{Ctr}$-independent function in differentiation of the organism (Suppl. Fig. 5).

We successfully generated chlamydial transformants with an inducible knockdown system to repress $ClpP2X_{Ctr}$ expression. To date, this study is the first of its kind in *Chlamydia* to knock down genes that are essential, highlighting the utility of CRISPRi in studies of chlamydial biology while providing insight into possible $ClpP2X_{Ctr}$ function. Notably, we observed a large decrease in IFU production coupled with an increase in plasmid loss after inhibition of *clpP2X* expression (Fig. 5). These effects were not observed when targeting a non-essential gene. Of note, penicillin does not kill chlamydiae but blocks cell division (63, 64), which keeps the organism transcriptionally in an RB-like state (65). This suggests that knocking down an essential gene(s) puts selective pressure on the chlamydiae to lose the plasmid encoding the CRISPRi system. This has important ramifications for long-term experiments and functional analyses. Nevertheless, the CRISPRi system represents a significant advance for our ability to study essential systems in this obligate intracellular bacterium.

In conclusion, we have demonstrated the importance of the $ClpP2X_{Ctr}$ system to chlamydial development, but many questions remain unanswered. These include why $ClpP2_{Ctr}$ and $ClpX_{Ctr}$ may serve independent purposes and what substrates this system may be targeting. Additionally, we need to identify any cofactors, chaperones, adaptor proteins, or a lack thereof that may be pertinent to this system. We plan to dissect the structural motifs of $ClpP2_{Ctr}$ and

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379 $ClpX_{Ctr}$ to determine if any of the noted differences from other bacterial Clp proteins may alter
380 activity, which may aid in our goal of further functional assessment. Finally, we need to continue
381 experimentation to address our overarching hypothesis that protein turnover plays a role in
382 chlamydial differentiation, and that the Clp system is a significant aspect of this model. Overall,
383 we conclude that the chlamydial $ClpP2X_{Ctr}$ system is critical to the development of these
384 obligate intracellular bacteria.

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Materials and Methods:

Strains and Cell Culture: The human epithelial cell line HEp2 was used for the overexpression assays, gDNA and protein extractions, and antibiotic studies. McCoy mouse fibroblasts were used for chlamydial transformation, and human epithelial HeLa cells were used for plaque purification. All of these cell lines were passaged routinely in Dulbecco's Modified Eagle's Medium (DMEM, Gibco/ThermoFisher) and 10% FBS (Sigma; St. Louis, MO) and verified to be mycoplasma-free using LookOut® Mycoplasma PCR Detection Kit (Sigma). Density gradient purified *Chlamydia trachomatis* L2/434/Bu (ATCC VR902B) EBs were used for the antibiotic studies. *C. trachomatis* serovar L2 EBs (25667R) naturally lacking the endogenous plasmid were prepared and used for transformation [see (66)].

Bioinformatics Analysis: Gene sequences of *Chlamydia trachomatis* were obtained from STDGen database (http://stdgen.northwestern.edu) or KEGG Genome Browser (67-69). RefSeq protein sequences from *Escherichia coli, Bacillus subtilis, Mycobacterium tuberculosis, Staphylococcus aureus,* and *Pseudomonas aeruginosa* were acquired from the NCBI protein database (https://www.ncbi.nlm.nih.gov/guide/proteins/). ClpX pairwise protein alignments to find sequence identity were performed using NCBI Protein BLAST function (https://blast.ncbi.nlm.nih.gov/Blast.cgi) (70). Multiple sequence alignments were performed using Clustal Omega (71) with default settings and were presented using Jalview Version 2 (72). PDB files for predicted monomeric 3D structures were acquired from the Phyre2 website (http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index) (73). Complexes were modelled using SWISS-MODEL available on the ExPASy server (74-77). Protein models and model alignments were rendered using the UCSF Chimera package from the Computer Graphics

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Laboratory, University of California, San Francisco (supported by NIH P41 RR-01081) (78). Docking analyses were performed with AutoDock Vina using the default parameter settings (45). Molecules were prepped using Dunbrack rotamer libraries (79, 80) to replace incomplete side chains and ANTECHAMBER for charge assignment and topology generation (81).

Plasmid Construction: A full list of the primers and plasmids used is included in the supplementary material. The Gateway® recombination system of cloning was used for plasmids for the Bacterial Adenylate Cyclase Two-Hybrid (BACTH) system (82). The genes were amplified from *Chlamydia trachomatis* L2 genomic DNA with added *attB* recombination sites. The PCR products were then incubated with a pDONR™221 entry vector (containing *attP* recombination sites) in the presence of BP Clonase II (Invitrogen) to insert the gene via flanking *attP* recombination sites and remove the *ccdB* insert, resulting in an entry vector containing the gene of interest flanked by *attL* sites. These constructs were transformed into DH5α chemically competent *E. coli* and plated onto kanamycin-containing LB agar. Plasmid was isolated and used for the LR reaction into one of three destination vectors (pST25-DEST, pSNT25-DEST, or pUT18C-DEST). The same entry vector for any given gene was used for all three LR reactions to insert into the destination vector. Entry vector and destination were incubated in a 1:1 ratio. DH5α *E. coli* were transformed with 2 μL of the reaction mix. Purified plasmid from an individual colony was sequence verified prior to use in the BACTH assay (see below).

Constructs for chlamydial transformation were created using the HiFi Cloning (New England Biolabs) protocol. Primers were designed to add a poly-Histidine (6xHis) tag to the gene of interest with the overlap to insert into the shuttle vector. Primers were generated using the NEBuilder® assembly tool available from New England BioLabs (http://nebuilder.neb.com).

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The backbone used was the pTLR2 derivative of the pASK plasmid (83). For the CRISPRi plasmid, the *S. aureus* dCas9 was PCR amplified from pX603-AAV-CMV::NLS-dSaCas9(D10A,N580A)-NLS-3xHA-bGHpA (a gift from Dr. F. Zhang; Addgene plasmid # 61594 (39)) and inserted into a derivative of pBOMB4-Tet::L2 (kind gift of Dr. T. Hackstadt, NIH; (84)) modified to weaken its ribosome binding site (Ouellette *in prep*). The gRNA cassettes were designed as previously described (38)), ordered as gBlock fragments from IDTDNA (Coralville, IA), and inserted into the BamHI site of the pBOMB4-Tet derivative encoding *Sa_dCas9* to produce, for example, the plasmid pBOMBLCRia::L2 (*clpP2X*). HiFi reactions were assembled according to the manufacturer's protocol. The reaction was transformed into DH10ß *E. coli*, and isolated plasmid was verified by restriction enzyme digest and sequencing by Eurofins Genomics. Sequence verified plasmids were transformed into *dam-/dcm- E. coli* (New England BioLabs) to produce demethylated plasmid, which was verified as described earlier prior to transformation into *C. trachomatis* (see below).

For mutation of ClpX Walker B motif, Q5 mutagenesis (New England BioLabs) was used. Primers were designed encoding the E187A mutation for PCR linearization of the plasmid. ClpX BACTH constructs were used as a template for the PCR amplification, and plasmids were re-circularized by KLD reaction. The resulting reactions were transformed into DH5α *E. coli* for plasmid production. Plasmids were isolated, and mutations were verified by Sanger sequencing (Eurofins Genomics) prior to use in the BACTH system. These plasmids also served as template for the PCR reactions to produce PCR products for insertion of the mutant *clpX* gene into the pTLR2 plasmid.

Strains created or used in this study are listed in the supplementary material. Transformed *E. coli* strains were maintained on LB agar plates, with antibiotics as necessary. To extract

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chlamydial genomic DNA, EBs were subjected to heat and proteinase K treatment prior to phenol:chloroform extraction (85). Sodium hydroxide lysis was utilized for the extraction of *E. coli* genomic DNA. For cloning into the pLATE31 plasmid, the aLICator LIC Cloning and Expression Kit 3 (Thermo Scientific) was used according to the manufacturer's specifications. Plasmids were first cloned into DH5α *E. coli* for plasmid propagation. Transformants were screened for inserts using colony PCR with Fermentas Master Mix (Thermo Scientific) and positive clones were grown for plasmid isolation (GeneJet Plasmid Miniprep Kit, Thermo Scientific). Sequence verified plasmids were then transformed into BL21(DE3) Δ*clpPAX E. coli* (55) for subsequent protein purification.

***Purification of Recombinant ClpX*:** His-tagged Ctr ClpX and Ctr $ClpX_{(E187A)}$ were purified from 500 mL cultures of BL21(DE3) Δ*clpPAX E. coli* transformed with the respective plasmid based on the protocol described in (17). Samples were induced with 0.5 mM IPTG and incubated with shaking for 20 hours at 18°C. Cultures were pelleted and frozen at -80°C prior to purifications. Samples were suspended in buffer A (25 mM Tris Base [pH 7.5], 300 mM NaCl, and 10 mM Imidazole), sonicated, bound to HisPur Cobalt Resin (Thermo Scientific), and washed in buffer A Proteins were eluted from the resin using buffer B (25 mM Tris Base [pH 7.5], 300 mM NaCl, and 300 mM Imidazole). Buffer exchange for ATPase assay buffer (25 mM HEPES [pH 7.2], 200 mM KCl, 20 mM $MgCl_2$, and 10% glycerol) was performed using a Millipore Amicon Ultra 15 filtration units (3 kDa cut-off). ClpX proteins were quantified using the Bio-Rad Protein assay, assessed for purity on 10% SDS-PAGE gels with Coomassie staining (Suppl. Fig. 6), and identified using anti-His-tag western blot. Blotting was performed using a mouse monoclonal

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anti-6x His antibody (1:1000; Millipore HIS.H8) and a goat anti-mouse IgG HRP conjugated secondary antibody (1:2000). Protein samples were aliquoted and stored at -80°C.

In Vitro *Analysis of ClpX Homo-Oligomerization:* 10 µg of purified protein was incubated at for 20 minutes at 37°C in oligomerization buffer (25 mM Tris Base [pH 7.5], 5 mM KCl, 5 mM $MgCl_2$, 1 mM DTT, and 1% glycerol) prior to mixing with a 5x native sample buffer (5 mM Tris [pH 6.8], 38 mM glycine, 0.06% bromophenol blue). Assays were analyzed on a BioRad MiniProtean 4-20% gradient gel for Native-PAGE. Gels were assessed using Coomassie staining.

***Assessment of ClpX ATPase activity* in vitro.**

A 49 µl reaction containing 1.5 µg of recombinant wild-type ClpX or $ClpX_{(E187A)}$ in ATPase assay buffer (see above) was preincubated for 10 minutes at room temperature without ATP. Next, ATP dissolved in ATPase assay buffer was added to 1 mM giving a final volume of 50 µl, and the reaction was incubated at 30º C for 2 hours. After the 2 hours, 200 µl of BIOMOL Green reagent (Enzo Life Sciences) was added and incubated at room temperature for 20 minutes. The absorbance of each reaction was then measured at 620nm using a BioTek Synergy HT plate reader. Reactions were performed in duplicate at least four times with at least two independent protein preparations.

Determining Protein-Protein Interactions with the BACTH System: The Bacterial Adenylate Cyclase Two-Hybrid (BACTH) assay was utilized to test interactions between wild-type and mutant ClpX (86). The genes of interest are translationally fused to one of either subunit,

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denoted as T18 and T25, of the *B. pertussis* adenylate cyclase toxin, which can complement adenylate cyclase deficient (Δ*cya*) DHT1 *E. coli*. Wild-type and mutant *clpX* genes cloned into one of the pST25, pSNT25, or pUT18C Gateway® vectors was tested for both homotypic and heterotypic interactions (9, 82). Plasmids from each background were co-transformed into chemically competent DHT1 *E. coli*, which were plated on a double antibiotic minimal M63 medium selection plate supplemented with 0.5 mM IPTG for induction of the protein, 40 μg/mL Xgal, 0.04% casein hydrolysate, and 0.2% maltose. Leucine zipper motifs were used for controls in pKT25 and pUT18C backgrounds on the appropriate antibiotic selection plates because these have been previously shown to interact (87). Blue colonies, indicative of positive interaction, were screened using the β-galactosidase assay. Random positive colonies were selected and grown in M63 minimal media with the appropriate antibiotics. 0.1% SDS and chloroform were used to permeabilize the bacteria prior to addition of 0.1% o-nitrophenol-β-galactoside (ONPG). 1 M $NaHCO_3$ was used to stop the reaction after precisely 20 minutes of incubation at room temperature. Absorbance at the 405 nm wavelength was recorded and normalized to bacterial growth (OD_{600}), dilution factor, and time (in minutes) of incubation prior to stopping the reaction. Totals were reported in relative units (RU) of β-galactosidase activity.

Chlamydial Transformation: The protocol followed was a modification of the method developed by Mueller and Fields (88) and as previously described (17). For transformation, 10^6 *C. trachomatis* serovar L2 EBs (25667R) naturally lacking the endogenous plasmid were incubated with 2 μg of unmethylated plasmid in a volume of 50 μL $CaCl_2$ at room temperature for 30 minutes. Reaction volume was sufficient for one well of a six well plate of McCoy mouse fibroblasts. Transformants were mixed with 1 mL of HBSS and added to 1 mL of HBSS in a six

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well plate. The plates were centrifuged at room temperature for 15 minutes, 400 xg. The plate was then incubated at 37° C for 15 minutes. After incubation, the HBSS was aspirated and replaced with antibiotic-free DMEM+10% FBS. 8 hours post-infection, the media was replaced with DMEM containing 1 µg/mL cycloheximide and 1 U/mL penicillin. Cells infected with transformants were passaged every 48 hours until a population of penicillin resistant bacteria was established. EBs were harvested and frozen in sucrose/phosphate (2SP; (66)) solution at -80° C.

Determining the Effect of Overexpression of Wild-Type and Mutant Clp Proteins via Immunofluorescence and Inclusion Forming Unit Analysis: *C. trachomatis* transformants containing plasmids encoding the 6xHis-tagged protein of interest were used to infect a confluent monolayer of HEp2 cells. Penicillin treatment was maintained throughout the duration of the infection. At 10 hpi, samples were induced or not with 10 nM anhydrotetracycline (aTc). At the given timepoints, three wells of a 24 well plate were scraped in 2SP, vortexed with three 1 mm glass beads, and frozen at -80° C. At the same timepoint, a coverslip was fixed in 3.25% formaldehyde and 0.025% glutaraldehyde for two minutes, followed by permeabilization with cold 90% methanol for one minute. Coverslips were labeled with primary goat anti-major outer membrane protein (MOMP; Meridian, Cincinnati, OH), rabbit anti-6xHis (Abcam, Cambridge, MA), and DAPI. Appropriate donkey secondary antibodies were used (Invitrogen, Carlsbad, CA). Images were acquired on an Axio ImagerZ.2 equipped with Apotome.2 optical sectioning hardware and X-Cite Series 120PC illumination lamp. Frozen IFU samples were titrated onto a fresh monolayer of HEp2s without antibiotics. At 24 hpi, samples were fixed with methanol for 10 minutes, stained for MOMP, and enumerated.

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***Genomic DNA Isolation and qPCR Enumeration of Genomic Equivalents*:** At 24 or 48 hpi, one well of a six well plate was scraped into the media overlay and pelleted at 17000 xg, 4° C for 15 minutes. Each sample was resuspended in 500 μL of cold PBS, frozen three times at -80° C, and processed using the Qiagen DNeasy Blood and Tissue Kit according to the manufacturer's specifications. DNA concentrations were assessed using a spectrophotometer prior to dilution down to 5 ng/μL. 5 μL of the resulting dilution was used for a 25 μL qPCR reaction volume using SYBR® Green PCR Master Mix (Applied Biosystems). Each reaction was performed in triplicate. A standard curve using Ctr L2 genomic DNA was generated for interpolation of sample Ct values. This experiment was performed three times for three biological replicates.

***Analysis of HctB Levels Upon Clp Overexpression*:** At 24 or 48 hpi, one well of a six well plate per test condition was rinsed twice with HBSS. To lyse the cells, 500 μL of denaturing lysis buffer (8 M Urea, 10 mM Tris, 2.5% 2-mercaptoethanol, 1% SDS) was added to each well and incubated for 15 minutes at room temperature. 300 units of Universal Nuclease (Pierce) per mL of lysis buffer was added immediately prior to addition to the wells. Following incubation, samples were centrifuged at 17000 xg, 4° C for 15 minutes to remove any insoluble material. Samples were quantitated using the EZQ Protein Quantitation Kit (Pierce). 50 μg of each sample was run in a 4-20% gradient SDS-PAGE gel (BioRad) and transferred to a PVDF 0.45 μm pore size membrane for 1 h at 300 mA. The membrane was probed using goat anti-MOMP (Meridian) and rabbit anti-HctB (generously provided by Dr. T. Hackstadt, NIH) primary antibodies followed by staining with donkey anti-goat 680 and donkey anti-rabbit 800 (LI-COR) secondary antibodies. The membrane was imaged on an Azure c600 imaging system. The channels were gray-scaled and equally contrast corrected, and the resulting images were used for integrated

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density measurement with FIJI software (89). To assess relative HctB levels, the HctB integrated density of each sample was normalized to its respective MOMP integrated density to avoid bias due to lower overall organism numbers. The ratios were then used to compare induced versus uninduced relative HctB levels. These experiments were performed three times for a total of three biological replicates.

Transmission electron microscopy (TEM) assessment of the effect of inactive Clp overexpression. Samples were infected and induced as previously discussed (see above). At 48 hpi, samples were fixed using 2% Glutaraldehyde, 2% Formaldehyde in 0.1M Sorensen's phosphate buffer, pH 7.2. Samples were then stained post-fixation in 1% Osmium Tetroxide in water for 1 hour. Samples were dehydrated in an Ethanol series 50%, 70%, 90%, 95%, 100% 3 changes of 100%, all steps 15 minutes each and were then soaked in Propylene Oxide 100% 3 changes for 15 minutes each. Samples were left overnight in a fume hood in a 1:1 mixture of Propylene Oxide and Embed 812. The following day the samples were placed in molds with fresh Embed 812 and polymerized overnight in an oven set at 65° C. Blocks were thin sectioned 90 nanometers thick on a Leica UC6 Ultramicrotome using a Diatome diamond knife. Sections were placed on uncoated 200 mesh copper grids and stained with 2% Uranyl Acetate and Reynold's Lead Citrate. Sections were examined on a FEI Tecnai G2 TEM operated at 80Kv.

Confirmation of* clpP2X *and* incA *knockdown. Briefly, two wells of a six-well plate per condition were infected with pBOMBLCRia-*clpP2X* transformed CtrL2 at an MOI of 0.8. At either four or ten hpi, samples were or were not induced with 10 nM aTc. At each given timepoint, total RNA was collected using Trizol reagent (Invitrogen) and was extracted with

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591 chloroform as described previously (17, 85, 90-92). The aqueous layer was precipitated using
592 isopropanol, as per the manufacturer's instructions. Samples were DNase treated using the
593 TURBO DNA-free kit (Ambion), and 1 µg of the resulting RNA was reverse transcribed using
594 SuperScript III reverse transcriptase (Invitrogen). Equal volumes of cDNA were loaded for each
595 qPCR reaction. To extract genomic DNA, one well per condition was harvested and processed
596 using the DNeasy blood and tissue kit (Qiagen) according to the manufacturer's instructions as
597 noted above. Samples were diluted to 5 ng/µL, and 5 µL of the resulting dilution was used per
598 qPCR reaction. cDNA and gDNA samples were quantified using 25 µL reactions with 2x SYBR
599 PowerUP Green Master Mix (Invitrogen) analyzed on a QuantStudio 3 (Applied Biosystems)
600 thermal cycler using the standard cycling conditions. A standard curve using purified wild-type
601 CtrL2 genomic DNA was generated for sample quantification. Data are displayed as the ratio of
602 cDNA to gDNA normalized to the 10h uninduced sample. For *incA* knockdown, HEp2 cells
603 were infected with the pBOMBLCRia-*incA* transformant, induced with 10 nM aTc as above, and
604 fixed at 24hpi with methanol. Cells were labeled with primary guinea pig anti-major outer
605 membrane protein (MOMP; kind gift of Dr. E. Rucks, UNMC), rabbit anti-Sa_dCas9 (Abcam,
606 Cambridge, MA), sheep anti-IncA (Dr. E. Rucks), and DAPI. Appropriate donkey secondary
607 antibodies were used (Invitrogen). Images were acquired on an Axio ImagerZ.2 equipped with
608 Apotome.2 optical sectioning hardware and X-Cite Series 120PC illumination lamp.
609
610 ***Determination of the effect of* clpP2X *knockdown on Ctr*.** 24-well plates of HEp2 cells were
611 infected at an MOI of 0.8 with either pBOMBLCRia-*clpP2X* or pBOMBLCRia-*incA*
612 transformed into CtrL2. Samples were induced or not at 4 hpi and were harvested, fixed, and
613 titered as previously described. Each titration was fixed using 4% formaldehyde and 0.025%

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glutaraldehyde to preserve GFP fluorescence. IFU counts of GFP positive inclusions are displayed as a percentage of the uninduced sample at the given timepoint. Plasmid retention for each condition is displayed as the percent of GFP positive to total number of inclusions for each condition.

Effect of ClpX-targeting compounds on chlamydial growth and host cell viability. Stocks of ClpX-specific inhibitor 334 and its derivative, 365, were synthesized as previously reported (44), resuspended at 25 mg/mL in DMSO, and frozen at -20°C. Methods for the synthesis, purification, and analysis of these compounds is available in Supplementary Information. A dose curve of treatment was performed to determine an inhibitory concentration of the compounds on Ctr, and 25 µg/mL was chosen (data not shown). For the 24 and 48 h samples, 500 µL of DMEM containing 25 µg/mL of the compounds were added at 8 hpi, and samples were harvested at the indicated timepoint. For the time of infection samples, compounds were added at 15 minutes post-infection and removed at 8 hpi. For the reactivation samples, DMEM containing the respective compound was added at 8 hpi, washed out three times with HBSS at 24 hpi, and then replaced with DMEM only for 24 additional hours prior to harvest. To determine the effect on preformed EBs, compound was added at 24 hpi, and samples were harvested at 48 hpi. To harvest, three wells of a 24-well plate were scraped into 2SP, vortexed with 3 mm glass beads, and frozen at -80^{0} C. Samples were titrated onto a fresh monolayer of HEp2 cells with no treatment for enumeration.

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Figure Legends

Figure 1: Bioinformatic analysis of chlamydial ClpX supports its role as a AAA+ ATPase. (A) Multiple sequence alignment of chlamydial ClpX with the ClpX orthologs of various other bacteria. Ec = *Escherichia coli*, Ctr = *Chlamydia trachomatis*, Bs = *Bacillus subtilis*, Mtb = *Mycobacterium tuberculosis*, Pa = *Pseudomonas aeruginosa*, Sa = *Staphylococcus aureus*. Alignment was performed using Clustal Omega with default settings and presented using Jalview version 2. Alignment was colored by % identity in shades of blue or as indicated below the alignment. (**B) 3D model** of ClpX was generated using SwissModel and presented in UCSF Chimera. Conserved motifs pseudo-colored as above in the MSA except the IGF loops, which are colored lime green. Two subunits of the hexamer were hidden for easier visualization into the complex. Top and bottom are representations following a 90° either clockwise or counterclockwise around the X axis. Of note, this model was generated using an ADP-bound form of ClpX as a template.

Figure 2: ClpX is a functional ATPase that forms the expected hexamer. (A) Native-PAGE assay of recombinant ClpX and $ClpX_{(E187A)}$. Expected hexameric size is approximately 283 kDa. **(B) ATP hydrolysis** end point assay using Biomol Green. Levels of detected phosphate are displayed on the Y axis. Error bars represent standard error and differences between samples are significant ($p < 0.05$, one-way anova). **(C) Bacterial Adenylate Cyclase Two Hybrid (BACTH)** assays showing pairwise, homotypic interaction of ClpX and $ClpX_{(E187A)}$ as well as heterotypic interaction of ClpX and $ClpX_{(E187A)}$. **(D) β-Galactosidase activity** of the BACTH interactions from (C), displayed in arbitrary units on the Y axis.

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Figure 3: Inactive Clp mutant overexpression negatively impacts Ctr at later timepoints. (A) Immunofluorescence assay (IFA) of ClpP2 and ClpX wild-type and inactive mutant overexpression at 24 and 48 hpi. Samples induced with 10 nM aTc at 10 hpi. Samples stained for MOMP (green), 6xHis (red), and DNA (blue). Scale bar = 10 μm. Images acquired on a Zeiss Apotome at 100x magnification. **(B-C) Recoverable inclusion forming units (IFUs)** of wild-type and mutant ClpP2 (B) or ClpX (C). Samples induced with 10 nM aTc at 10 hpi. IFUs recovered displayed as Log_{10}. Values represent the average of two independent experiments. **(D) IFA of ClpP2X** wild-type or inactive mutant operons at 24 and 48 hpi. Samples stained for MOMP (pink), FLAG (ClpP2, red), 6xHis (ClpX, green), and DNA (blue). Parameters as described in previous figure. **(E) IFU recovery assays** of ClpP2X wild-type and mutant overexpression. IFUs recovered displayed as Log_{10}.

Figure 4: Functional disruption of ClpX and ClpP2 perturbs chlamydial development, but likely in different manners. (A) Fold changes of detectable gDNA from 24 to 48 hpi for each strain. Samples induced at 10 hpi with 10 nM aTc. * = $p \leq 0.05$, ** = $p \leq 0.001$ by paired t-test. Values displayed as the average of three independent experiments with error bars representing standard deviation. **(B) Western blot** analysis of HctB levels at 48 hpi with and without overexpression. One well of a six well plate was lysed into 500 μL of denaturing lysis buffer. 50 μg of protein from the clarified lysate for each sample was loaded and run. Blots were probed for MOMP (IR680) and HctB (IR800). Grayscale blot shown is representative of three independent experiments. **(C) Quantified integrated** density of the staining from (B). HctB levels were normalized to MOMP levels in each sample to account for differences in bacteria in each sample.

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Values displayed as levels of induced to uninduced HctB/MOMP ratios for each strain. * = $p \leq 0.05$, ** = $p \leq 0.001$, ns = not significant by multiple comparisons t-test.

Figure 5: Knockdown of *clpP2X* expression negatively impacts *Chlamydia*. (A) Transcript levels upon knockdown of *clpP2X* by CRISPR interference. Data displayed are the average and standard deviation of three independent biological replicates of triplicate RT-qPCR reactions. Values are normalized to the 10h uninduced ClpP2 value for each experiment. Samples were induced using 10 nM aTc. **(B) Immunofluorescence staining** to confirm knockdown of IncA upon induction of dCas9 expression. Sa_dCas9 was induced or not at 4 hpi. Samples were harvested at 24 hpi and were stained for chlamydial MOMP (green), IncA (magenta), and DNA (Blue). Scale bar = 10 μm. **(C) Recoverable inclusion forming units (IFUs)** following induction of knockdown at 4 hpi. Values are presented on a Log_{10} scale percent of the respective uninduced titer. Error bars represent standard deviation between experiments. **(D) Plasmid retention** based on the ratio of GFP positive to total number of inclusions is displayed in percent for each condition. Error bars represent the standard deviations of three independent biological replicates.

Figure 6: Chemical disruption of ClpX function is highly detrimental to *Chlamydia*. (A) Immunofluorescence assay (IFA) of 24 hours post-infection (hpi). All drugs added at a final concentration of 25 μg/mL. Drugs were added at 8 hpi for the 24 h treatment samples. Drugs were added 15 minutes post-infection and removed at 8 hpi for the TOI: 8 h pulse samples. MOMP is stained in green, and DNA is stained with DAPI. Images were acquired on a Zeiss LSM800 microscope at 63x magnification. Scale bar = 10 μm. TOI = Time of Infection. **(B) IFA**

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of 48 hpi samples. Samples stained for MOMP (green) and DNA (blue). Scale bar = 10 μm. Drug was added at 8 hpi for 48 h and reactivation samples. Media removed, drug washed out, and media with drug added back to +Drug at 24 h and 48 h samples. Reactivation samples media replaced with DMEM, no drug. **(C) Recoverable inclusion forming units (IFUs)** from the indicated conditions. Totals present as Log_{10} IFUs recovered. Standard deviation displayed on graphs as error bars.

Supplemental Figure Legends

Figure S1: Transmission electron microscopy of inactive Clp overexpression. (A) Representative $ClpP2_{S98A}$ uninduced or induced samples at 48 hpi. Samples were induced or not with 10 nM aTc at 10 hpi and were fixed and processed at 48 hpi. Arrows indicate abnormal forms in the induced samples. Scale bar = 2 μm. **(B) Representative $ClpX_{E187A}$ uninduced or induced** samples at 48 hpi. Samples treated, fixed, and processed as previously discussed. Arrows indicate abnormal forms with intrabacterial aggregates. Scale bar = 2 μm. **(C and D) Zoomed images of boxed regions from $ClpX_{E187A}$ uninduced** samples at 48hpi.

Figure S2: RT-qPCR of *clpP1*, *euo*, and *omcB* upon *clpP2X knockdown*. Data shown are the average of three biological replicates, each with three technical replicates. Values are normalized to the 10h timepoint for each respective gene. Error bars represent standard deviation. Samples induced as indicated with 10 nM aTc.

Figure S3: Docking simulation of the ClpX inhibitor 334 on a ClpX model. (A) PDB structure of 334. Inset is the 2D structure of the drug. **(B) Ribbon model** of docked 334 within

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the ClpX hexamer. The best scoring model is shown. Only the two ClpX subunits making contact with the model are shown (A in gray, B in seafoam green). The Walker A motif (red), Walker B motif (purple), sensor 1 motif (dark green), and sensor 2 motif (orange) of subunit A are colored for visualization. The arginine finger is labeled light green of subunit B. The center picture is oriented as outside of the complex looking inward, and the other images are rotations as indicated. **(C) Surface rendering** of the ClpX subunits with docked 334 are shown with coloration as in (B).

Figure S4: Docking simulation of the ClpX inhibitor 365 on a ClpX model. (A) PDB structure of 365. Inset is the 2D structure of the drug. **(B) Ribbon model** of docked 365 within the ClpX hexamer. The best scoring model is shown. Only the two ClpX subunits making contact with the model are shown (A in gray, B in seafoam green). The Walker A motif (red), Walker B motif (purple), sensor 1 motif (dark green), and sensor 2 motif (orange) of subunit A are colored for visualization. The arginine finger is labeled light green of subunit B. The center picture is oriented as outside of the complex looking inward, and the other images are rotations as indicated. **(C) Surface rendering** of the ClpX subunits with docked 365 are shown with coloration as in (B).

Figure S5: Proposed model for ClpP2X function in *Chlamydia*. An RB ultimately has two fates: differentiation or replication. Based on our data showing the impact of ClpP2 disruption on the developmental cycle, we hypothesize that ClpP2 may play a role in triggering either event based on its degradative target at either timepoint. Furthermore, we posit that ClpX may play a

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substantial role in EB organization, given that ClpX affects recoverable IFUs without reducing the amount of HctB produced.

Figure S6. Example ClpX protein purifications. Recombinant, 6x His-tagged ClpX/ClpX$_{(E187A)}$ were purified using cobalt-based immobilized metal affinity chromatography. Samples were quantified and 1 and 5 µg aliquots were run on 10% SDS-PAGE followed by staining with Coomassie brilliant blue. Three ClpX and two ClpX$_{(E187A)}$ purification were performed using BL21(DE3) Δ*clpPAX E. coli*.

Supplemental Table 1. List of primers, plasmids, and strains used in this study.

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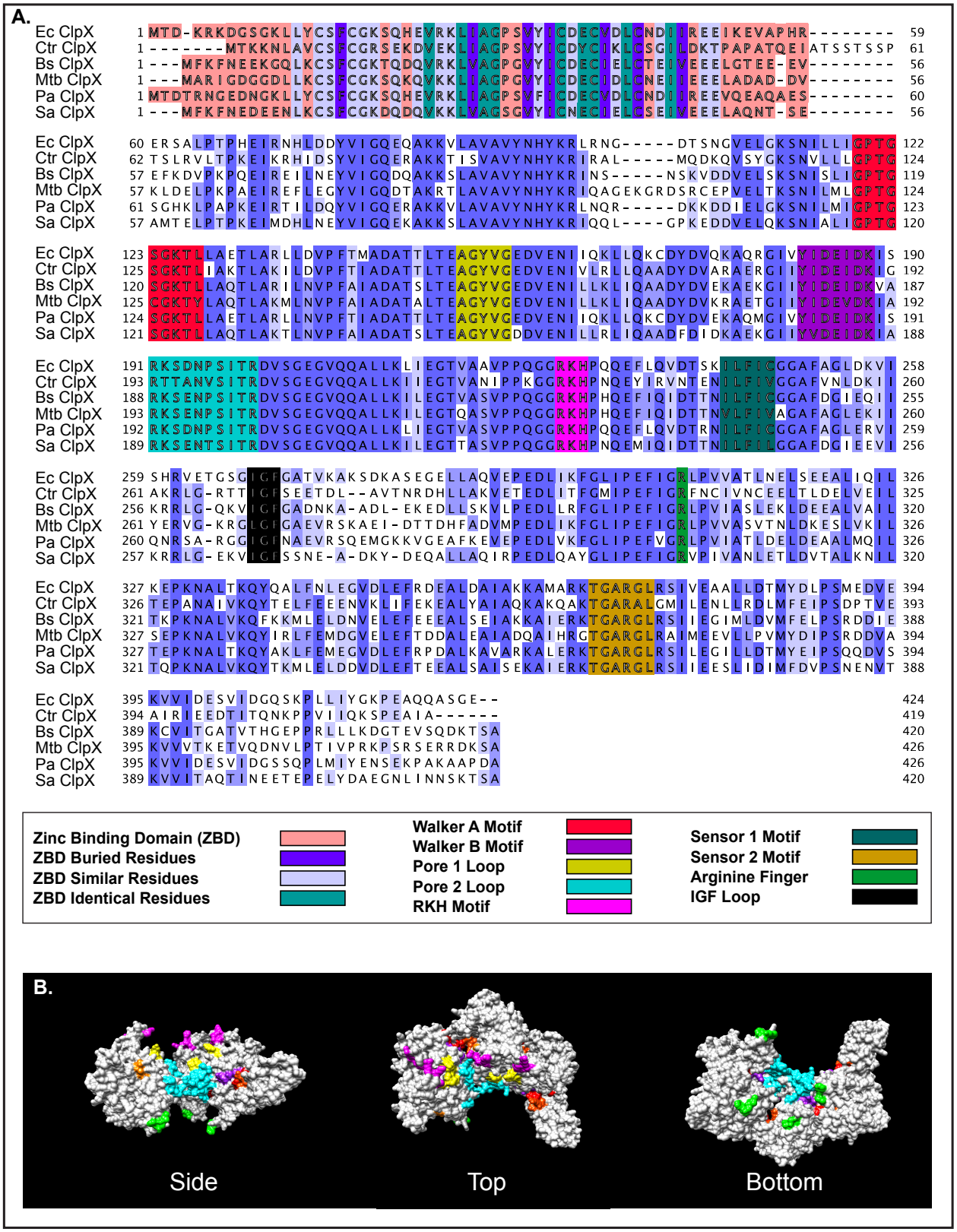

Fig. 1

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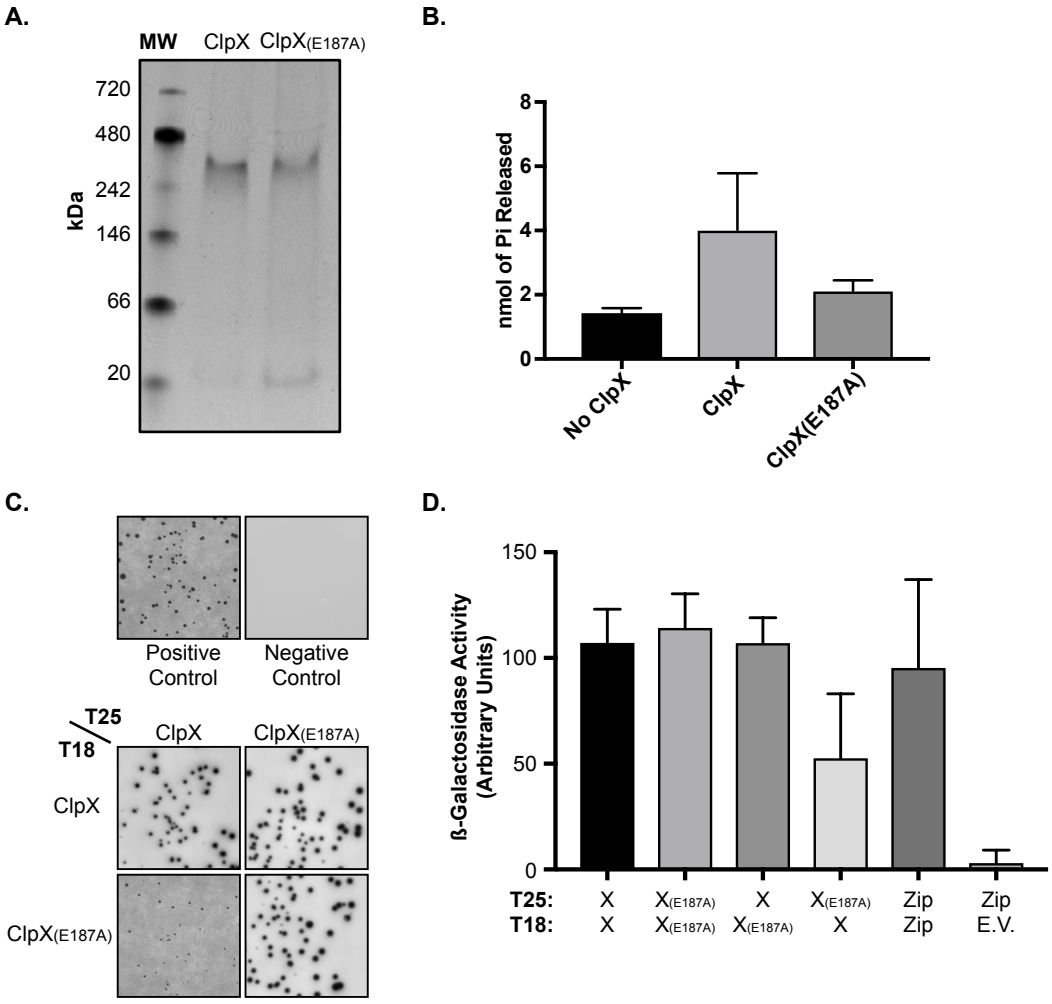

Fig. 2

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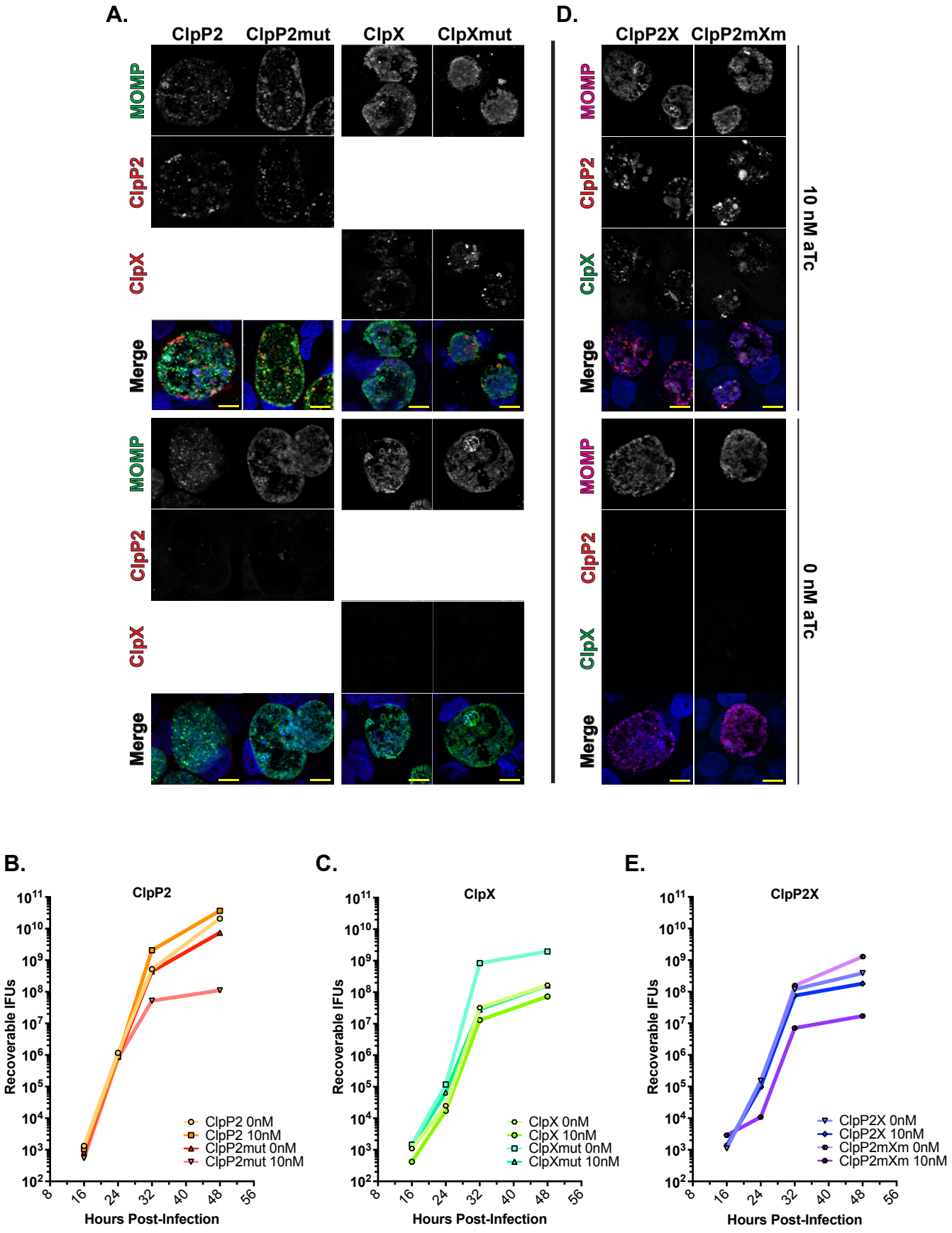

Fig. 3

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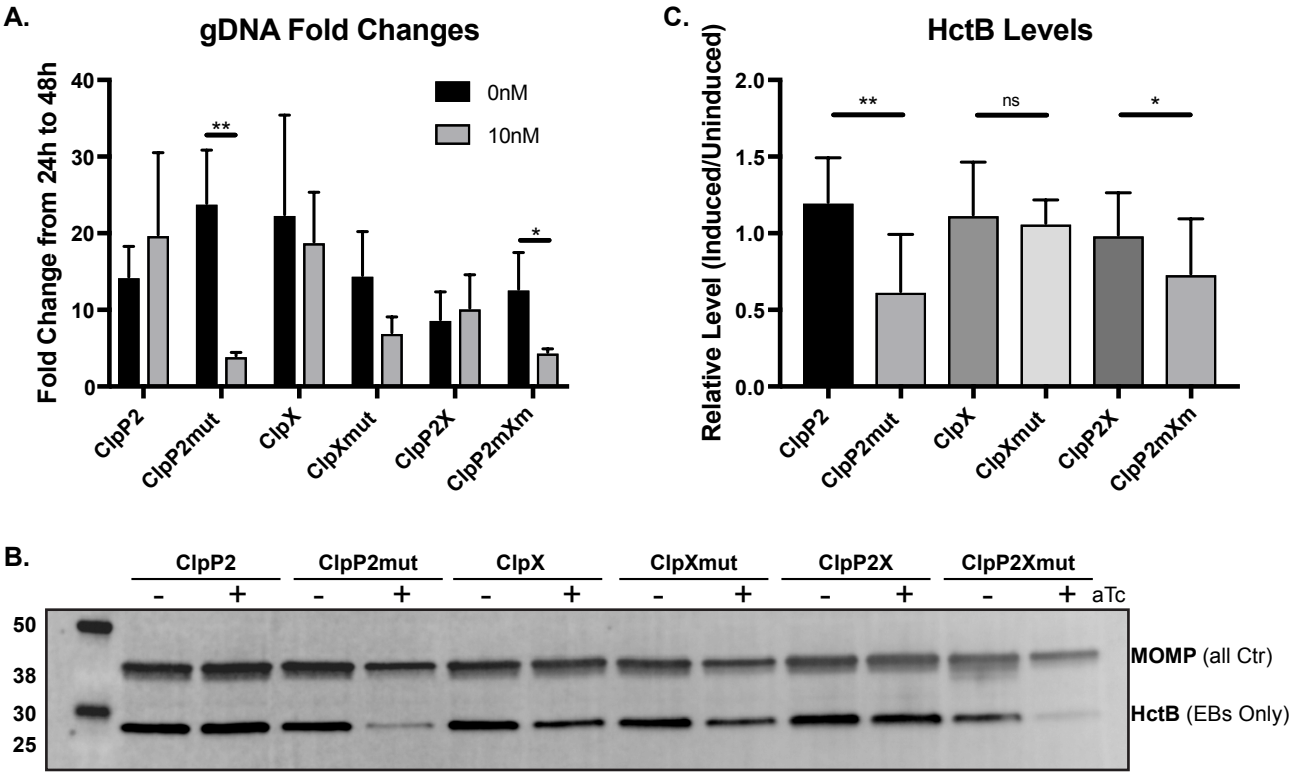

Fig. 4

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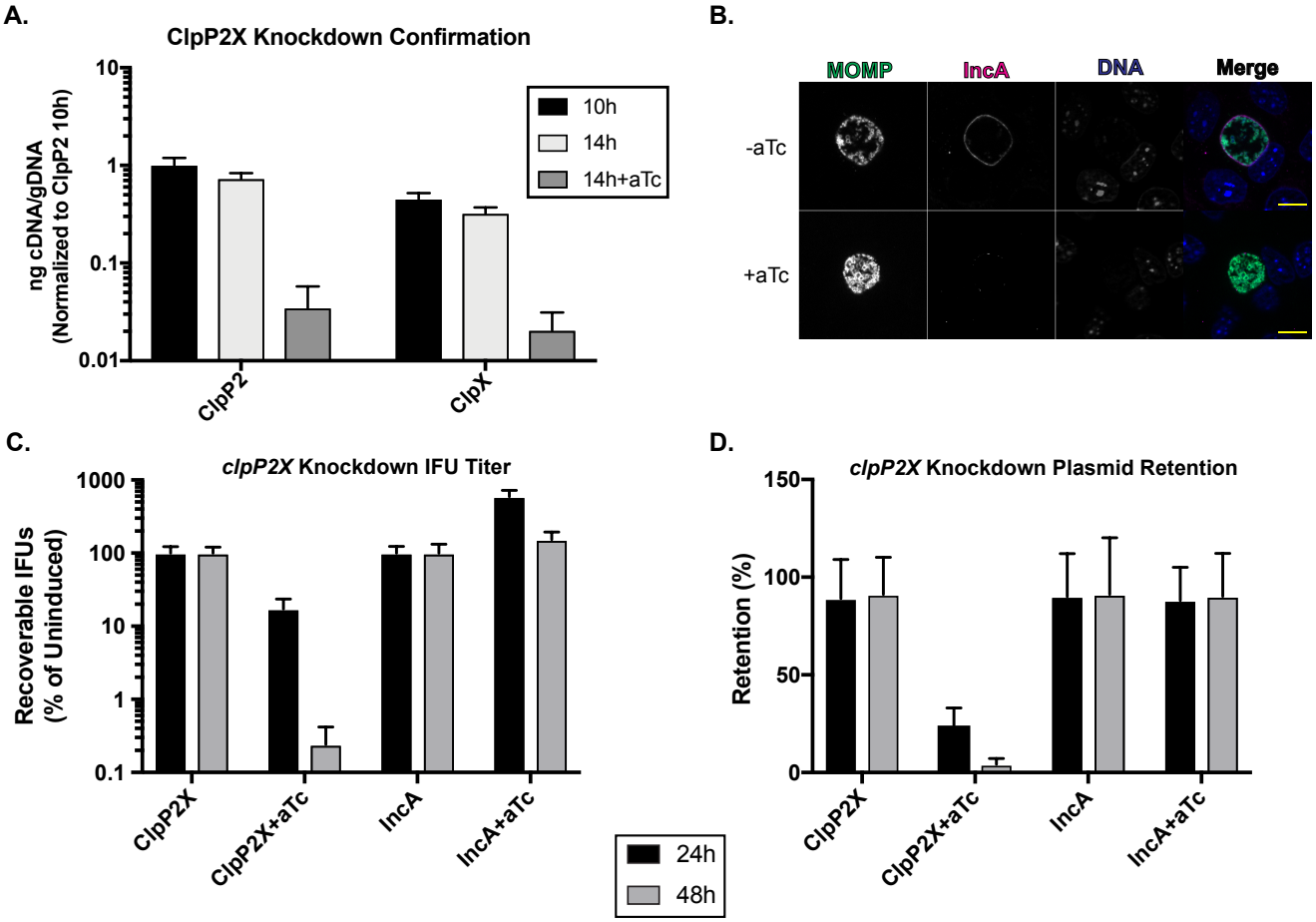

Fig. 5

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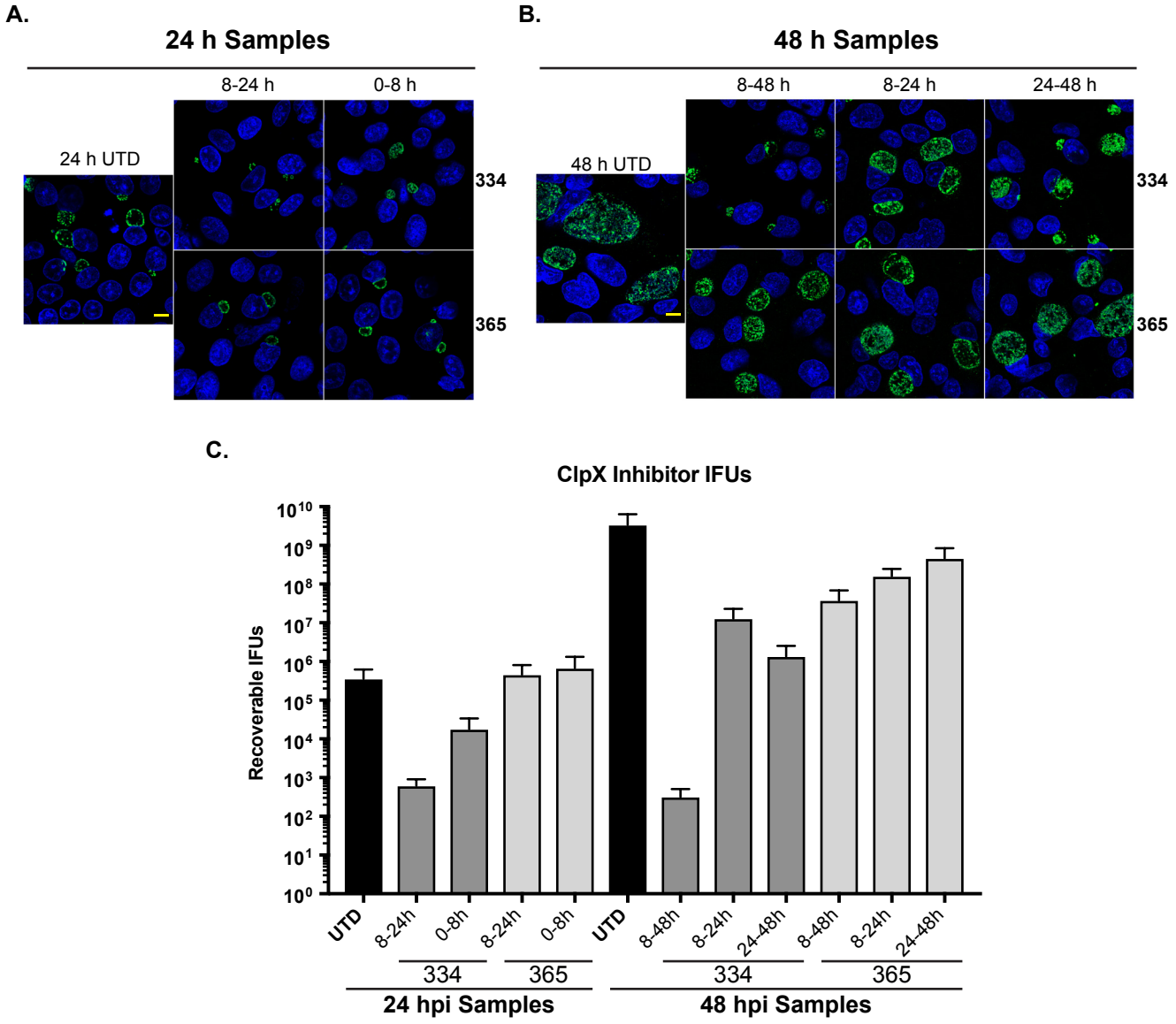

Fig. 6