Regulation of invasion-associated actin dynamics by the *Chlamydia trachomatis* effectors TarP and TmeA

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Abstract:

The obligate intracellular pathogen *Chlamydia trachomatis* manipulates the host actin cytoskeleton to assemble actin-rich structures that drive pathogen entry. This actin remodeling event exhibits relatively rapid dynamics that, through quantitative live-cell imaging, was revealed to consist of three phases – a fast recruitment phase which abruptly transitions to a fast turnover phase before resolving into a slow turnover of actin, indicating the end of actin remodeling. Here, we investigate *Chlamydia* invasion in the context of actin dynamics. Efficient invasion was associated with robust actin remodeling kinetics, which was linked to signaling from the type-III secreted effectors TarP and TmeA, and the actin nucleating activities of formin 1 (Fmn1) and Arp2/3. Stable recruitment of Fmn1 and Arp2/3 was dependent upon TarP and/or TmeA, although TarP signaling was responsible for the majority of Fmn1 and Arp2/3 recruitment. Rapid actin kinetics were due in part to a collaborative functional interaction between two different classes of actin nucleators – formins, including formin 1 and the diaphanous-related formins mDia1 and mDia2, and the Arp2/3 complex. Inhibition of either formin or Arp2/3, or deletion of TarP and TmeA, prevented this collaboration and resulted in attenuated actin kinetics and invasion efficiency. Collectively, these data support a model wherein TarP and TmeA signaling are core components of actin remodeling that operate via stable recruitment of formin and Arp2/3. At the population level, the kinetics of recruitment and turnover of actin and its nucleators were linked. However, reanalysis of the data at the level of individual elementary bodies showed significant variation and a lack of correlation between the kinetics of recruitment and turnover, suggesting that accessory factors variably modify actin kinetics at individual entry sites. In summary, efficient chlamydial invasion is an effector-driven process that requires a specific profile of actin recruitment which arises following collaboration between formin and Arp2/3.

Short Title: TarP and TmeA coordinate collaboration between Formin 1 and Arp2/3 during invasion

Author Summary:

The obligate intracellular pathogen *Chlamydia trachomatis* relies upon manipulation of the host actin cytoskeleton to drive its entry into host cells, such that impairment of actin dynamics attenuates *Chlamydia* invasion. Collaboration between two classes of actin nucleators, formin and Arp2/3, are known to enhance actin recruitment and turnover; we found that stable recruitment of both proteins to the signaling complex established by the type III secreted effectors, TarP and TmeA, were important for pathogen internalization. Furthermore, Formin 1 and Arp2/3 are co-recruited to sites of entry, and pharmacological inhibition of either actin nucleator impaired recruitment of the other, indicating a functional cooperation between branched and filamentous actin nucleation within pathogen entry sites. Disruption of this cooperation negatively impacted both actin dynamics and *Chlamydia* internalization, indicating that TarP- and TmeA-dependent entry of *Chlamydia* into non-phagocytic cells operates through the recruitment and activation of Arp2/3 and Formin 1. Finally, kinetic analysis of actin recruitment and turnover revealed that these processes were independently regulated, in addition to implicating the presence of local factors that fine-tune actin dynamics and subsequent invasion.

Introduction

Infections caused by the obligate intracellular bacterium *Chlamydia trachomatis* are both the leading cause of preventable blindness and the most prevalent bacterial form of sexually transmitted disease worldwide [1]. *Chlamydiae* feature a biphasic developmental cycle divided between metabolically inactive elementary bodies, which infect host cells, and metabolically active reticulate bodies, which replicate in a membrane-bound vacuole known as an inclusion [2]. Invading elementary bodies initially form a reversible electrostatic attachment onto heparin sulfate proteoglycans on the host cell surface [3,4]. Shortly after, *Chlamydia* engages and activates a multitude of host cell receptors while simultaneously delivering an array of bacterial effectors by a type III secretion system [5–7]. Activation of host receptors and delivery of bacterial effectors during invasion allow *Chlamydia* to exploit regulatory components of the actin cytoskeleton like Arp2/3, Rho GTPases, and vinculin amongst others [5,7–11]. By manipulating cytoskeletal regulators, *Chlamydia* induces the formation of actin-rich microstructures on the cell surface that facilitate engulfment of the pathogen [12,13]. These structures can adopt a variety of configurations, including phagocytic cups, membrane ruffles, filopodia, and hypertrophic microvilli [9,14]. A distinguishing characteristic of structures generated by *Chlamydia* during invasion is their rapid and highly localized assembly [12]. Whereas *S. typhimurium* and *R. parkeri* recruit actin in a diffuse pattern over the span of 5-15 minutes [15–17], actin recruitment and localized assembly of invasion-associated structures by *Chlamydia* is completed within two minutes [18].

Actin polymerization at the invasion site occurs is due in large part to the secretion of the bacterial effectors TarP and TmeA, which jointly coordinate the assembly of a robust actin remodeling network at *Chlamydia* entry sites. TarP and TmeA accomplish this task in part by their shared ability to recruit the actin nucleator Arp2/3 during invasion. Arp2/3 is a complex of proteins which, upon activation, enhances the rate of actin polymerization by nucleating branched actin filaments along a pre-existing “mother” filament [19,20], and the resulting branched network in turn increases the number of sites for Arp2/3

binding and branched nucleation [21]. TarP and TmeA activate the Arp2/3 complex via downstream activation of the nucleation promoting factors WAVE2 and N-WASP, respectively [9,22–24]. Signaling through WAVE2 and N-WASP have each been shown to be important for *Chlamydia* engulfment, as targeted disruption of either protein attenuated pathogen uptake [9,23,24]. Furthermore, siRNA knockdown of WAVE2 prevented the recruitment of actin at entry sites, highlighting the importance of Arp2/3 activation and subsequent actin remodeling in *Chlamydia* pathogenesis [9].

The actin remodeling network generated by *Chlamydia* enable the assembly of invasion-associated structures which are comprised entirely or in part by elongated bundles of filamentous actin. A class of actin nucleators known as formins participate in the assembly of actin-rich structures by facilitating rapid incorporation of actin monomers onto the barbed end of actin filaments [25]. In turn, these rapidly elongating filaments provide a highly localized protrusive force that gives rise to actin-rich structures such as filopodia and microvilli [26–28]. Broadly, formins can be divided into two subclasses: diaphanous-related formins (DRFs), which must be activated by Rho-GTPases before they can serve as actin nucleators, and non-DRF formins, which do not require activation to participate in actin filament elongation [29]. Once activated, formins dimerize and incorporate monomeric actin onto the barbed end of an actin filament. Formins have been demonstrated to participate in the invasion of bacterial pathogens including *L. monocytogenes*, *S. typhimurium*, and *B. burgdorferi* [30–32]. Likewise, invading *C. trachomatis* is known to interact with hypertrophic microvillar structures that are enriched with filamentous actin [12], raising the possibility that *Chlamydia* also utilizes formins during invasion.

The actin elongating properties of formins are known to synergize with the actin branching activity of Arp2/3, generating a dense network of branched and elongated actin filaments [33]. This collaborative interaction is essential for many cellular processes like cell motility, division, and phagocytosis [34,35], and also enhances the actin remodeling capabilities of invading pathogens. For instance, the spiraling filamentous protrusions formed by *B. burgdorferi* or the raised actin-rich pedestals of enteropathogenic

E. coli are both formed by collaboration between Arp2/3 and formin [36,37]. These pathogen-associated structures are reminiscent of the structures generated during *C. trachomatis* invasion, introducing the possibility that formin and Arp2/3 also act collaboratively during invasion to engulf the pathogen.

In addition to the multitude of proteins which regulate the polymerization of the actin cytoskeleton, a handful of other proteins coordinate the depolymerization of actin [38]. Typically, actin depolymerization factors target older actin filaments to generate monomeric actin, which in turn is utilized to generate new actin filaments [39]. Despite their role in maintaining actin homeostasis, the activities of host actin depolymerization factors are often counteracted by bacterial pathogens [40]. For instance, the *C. pneumoniae* TarP ortholog CPN0572 displaces the actin depolymerization factor ADF/cofilin to stabilize actin filaments during its invasion [41]. However, it is evident that actin turnover is necessary for *Chlamydia* internalization, as treatment with the actin stabilizing compound jasplakinolide prevents invasion [42]. Furthermore, actin recruitment observed in previous studies is highly transient [18], indicating that *Chlamydia* employs a mechanism that induces a shift from the recruitment and polymerization of actin toward an equally rapid depolymerization, which removes the dense network of filaments generated by *Chlamydia* during invasion. The details regarding how *Chlamydia* accomplishes actin depolymerization, or the signals that initiate this process remain unknown.

Given the importance of actin recruitment and turnover to chlamydial invasion, we investigated this process by quantifying the relative contribution of TarP and TmeA to the overall actin dynamics during invasion. Actin dynamics associated with efficient invasion consists of three phases: rapid recruitment, a brief fast turnover phase, and a lingering slow turnover phase. We observed that *Chlamydia* strains lacking either TarP or TmeA invaded epithelial cells less efficiently and exhibited altered actin dynamics; direct comparison of the two mutants revealed a greater defect in actin dynamics in the TarP knockout strain. The two mutants also exhibited quantifiable reduction in the recruitment and turnover kinetics for Arp2/3 and formin 1 (Fmn1), thus accounting for the significant differences in actin dynamics and subsequent

invasion. Using target-specific pharmacological inhibitors that affected actin nucleation, but not association with nucleation promoting factors and/or actin filaments, we were able to demonstrate the mutual recruitment of Arp2/3 and Fmn1 in an actin polymerization-dependent manner. Inhibition of one was sufficient to inhibit recruitment of the other, indicating that their presence at the sites of invasion was due to their respective abilities to bind and nucleate filamentous actin. Collectively analyzing dynamics data for actin, Arp3, and Fmn1 at the single-elementary body level also showed that recruitment and turnover rates were not strictly interdependent, hinting at additional molecular determinants of invasion efficiency. In summary, this report established a functional hierarchy of TarP and TmeA which operates to regulate actin dynamics, an observation that was not readily apparent from invasion efficiency quantification alone. As such, we conclude that the rapid and highly localized actin network generated by *Chlamydia* during invasion emerges as a consequence of collaboration between the activities of Arp2/3 and formin following their recruitment by TarP and TmeA.

Results

Both TarP and TmeA are necessary for efficient invasion and optimal actin kinetics.

Rapid and robust recruitment of actin is a hallmark of *Chlamydia* invasion, which is coordinated in part by the secreted effectors TarP and TmeA via their ability to recruit actin modulatory proteins during invasion. Recently, it was shown that TarP and TmeA synergistically enhance the in-vitro rate of actin polymerization [24]. Genetic deletion of TarP (Δ TarP) or TmeA (Δ TmeA) attenuates pathogen entry, which was rescued by cis-complementation of *ct456/tarp* (*cis*-TarP) or *ct694/tmea* (*cis*-TmeA). On the basis of these observations, it was proposed that synergy between TmeA and TarP is an important determinant of both robust actin kinetics and efficient invasion. To evaluate this claim, we quantified the rate and intensity of actin recruitment at the entry sites of wild-type, single-knockout (Δ TarP, Δ TmeA), cis-complemented (*cis*-TarP, *cis*-TmeA), or double-knockout (Δ TmeA/ Δ TarP) *Chlamydia* strains (Fig. 1, Video

S1). Comparisons of actin recruitment between strains were derived by calculating their respective fold recruitment values, which measures the fold increase of fluorescence intensity relative to the immediate background. A detailed account of this process can be found in the supplementary data (Fig. S1). While all strains retained the ability to recruit actin, we noted that the intensity of actin recruitment was diminished upon deletion of TarP and/or TmeA (Fig. 1A,B). Wild-type and *cis*-complemented strains of *Chlamydia* recruited actin at an average intensity of 18-fold above background, which was reduced by 5-fold following TmeA deletion and 12-fold following TarP deletion. Interestingly, the peak intensity of actin recruitment at Δ TmeA/ Δ TarP entry sites (4.86 fold $\pm$ 0.68) was only marginally reduced relative to Δ TarP (6.27 fold $\pm$ 1.10), suggesting that TmeA signaling alone is insufficient to generate robust actin kinetics and that TarP accounts for the majority of the actin signal intensity during invasion. Despite this, and in agreement with earlier studies, we found that deletion of either TmeA or TarP significantly reduced the efficiency of *Chlamydia* internalization (Wild-type=76.2%, Δ TmeA=50.4%, Δ TarP=42.1%), and that deletion of both effectors worsened this defect (Δ TmeA/ Δ TarP=29.6%) (Fig. 1C). Collectively, these data indicate that strains lacking TarP and/or TmeA are deficient in actin recruitment, which is linked to poor invasion efficiency relative to either wild-type or *cis*-complemented strains.

Recruitment and turnover of actin are slowed by deletion of TarP and/or TmeA

In addition to reducing the intensity of actin recruitment, we also noted that the overall actin recruitment profiles of Δ TarP and Δ TmeA strains differed from wild-type *Chlamydia*. In particular, both Δ TarP and Δ TmeA/ Δ TarP exhibit atypically transient actin recruitment, while Δ TmeA produces an intermediate profile with slightly lower intensity and duration (Fig. 1B). These observations, coupled with reduced invasion efficiency following TarP or TmeA deletion (Fig. 1C), indicate a link between actin kinetics and invasion efficiency. We observed that the recruitment profile of actin exhibited three distinct phases: i) recruitment, in which actin rapidly accumulates, ii) fast turnover, in which actin is rapidly lost from the

sites of invasion, likely through depolymerization and iii) slow turnover, in which actin is gradually depolymerized over an extended time period (Fig. 1B). The entry and exit points for each phase were determined by plotting the first derivative values corresponding to the velocity of recruitment and turnover for each timepoint and is discussed in greater detail in the supplemental data (Fig. S2).

To compare the kinetics of actin recruitment and turnover between conditions, we employed linear regression to calculate the slope, representing the rate of recruitment and turnover for each recruitment event (Fig. S2,3), and mapped these values onto a violin plot to visualize the distribution of rates for each strain (Fig. 1D,E, Table S1). Although wild-type and *cis*-TarP strains exhibited comparably rapid actin recruitment (WT=0.287 fold/sec, *cis*-TarP=0.233 fold/sec), we observed a slower rate of actin recruitment in *cis*-TmeA (0.178 fold/sec), which is consistent with the observation that *cis*-TmeA achieves peak actin intensity roughly 20 seconds later than wild-type or *cis*-TarP strains (Fig. 1B). Deletion of either TmeA or TarP attenuated actin recruitment kinetics by 2-fold and 5-fold, respectively, relative to wild-type *Chlamydia* (Δ TmeA=0.178 fold/sec, Δ TarP=0.054 fold/sec). Actin kinetics were further restricted by simultaneous deletion of both TmeA and TarP (Δ TmeA/ Δ TarP=0.031 fold/sec), yielding a nearly 10-fold reduction in the rate of actin recruitment. Altogether, these data suggest that optimal rapid actin recruitment is an effector-driven process that relies on both TarP and TmeA. Furthermore, we observed that actin kinetics were more severely attenuated in strains that lack TarP (Δ TarP, Δ TmeA/ Δ TarP), suggesting that TarP is the major determinant of actin recruitment kinetics, and that TmeA has a minor role in rapid actin recruitment.

Despite exhibiting variability in recruitment kinetics, we noted that the actin turnover rate for wild-type, *cis*-TmeA, and *cis*-TarP were all comparably fast (Wild-type=0.082 fold/sec, *cis*-TmeA=0.104 fold/sec, *cis*-TarP=0.117 fold/sec) (Fig. 1E). Interestingly, we observed that deletion of either TmeA or TarP roughly halved the turnover rate of actin (Δ TmeA=0.068 fold/sec, Δ TarP=0.043 fold/sec) compared to wild-type, suggesting that TmeA and TarP influence both actin recruitment and turnover. Notably, TmeA

deletion exerted a greater impact on the kinetics of actin turnover than recruitment, whereas TarP deletion greatly impacted both actin recruitment and turnover. Furthermore, the duration of actin turnover was substantially reduced in strains that lack TarP, lasting on average for 120 seconds before actin intensity returned to near-background levels (Fig. S4). In contrast, turnover lasted for an average of 380 seconds in wild-type and *cis*-complemented strains, and 240 seconds for the Δ TmeA strain. Collectively, these data support a model wherein TarP and TmeA determine the quality of actin dynamics.

Thus far, we have identified four relevant attributes of actin dynamics that exhibit variability at the population level upon deletion of TarP and/or TmeA: i) recruitment rate, ii) recruitment intensity iii) turnover rate, and iv) turnover duration. To clarify how recruitment and turnover are regulated at the single EB level, we calculated all four attributes for each individual recruitment event represented in the population-level data. Using these values, we conducted a series of pairwise correlation analyses to identify the relationship between attributes (Table 1). Surprisingly, despite the link between rapid actin recruitment and rapid turnover present at the population level, we noted that individual EBs exhibited moderate-to-weak correlation between the rate of recruitment and turnover, implying a level of independence between the two phases. This could be due to the presence of predetermining factors that variably influence the recruitment and turnover rates of actin at each invasion event. Some examples include the local concentration of invasion-relevant translocated effectors, the density of pre-formed actin networks which serve as sites for initial or additional actin polymerization, or the abundance of signaling co-receptors. These local variations could result in variable actin dynamics at the individual level that become less apparent when analyzed at the population level.

Table 1: Pairwise correlation analyses of actin kinetics following deletion or *cis*-complementation of TarP and TmeA

	Recruitment Rate vs. Turnover Rate	Recruitment Rate vs. Intensity	Turnover Rate vs. Intensity	Turnover Duration vs. Intensity
Actin <i>cis</i> -TmeA	r= 0.108	r= 0.168	r= 0.446	r= 0.175
Actin Δ TmeA	r= 0.246	r= 0.277	r= 0.47	r= 0.068
Actin <i>cis</i> -TarP	r= 0.338	r= 0.568	r= 0.49	r= 0.183
Actin Δ TarP	r= 0.535	r= 0.177	r= 0.654	r= 0.364
Δ TmeA/ Δ TarP	r= 0.283	r= 0.673	r= 0.565	r= 0.21

Negligible	Weak	Moderate	Strong	Very Strong
0.00 - 0.19	0.20 - 0.39	0.40 - 0.59	0.60 - 0.79	0.8+

We also observed a consistently strong correlation between the rate of actin turnover and its corresponding peak intensity, which was not observed during the recruitment phase. This implies that the rate of turnover is dictated by the amount of polymerized actin, or the density of the actin network formed at the peak of recruitment. As a result, the duration of actin turnover is reasonably consistent among individual EBs despite exhibiting substantial variation in actin intensity, as indicated by weak correlation between turnover duration and actin intensity. Finally, we noted that Δ TarP EBs, and to a lesser extent Δ TmeA/ Δ TarP EBs, appear to be regulated distinctly from strains that possess TarP. In particular, Δ TarP exhibited a strong correlation between recruitment and turnover rates and moderate correlation between turnover duration and intensity (Table 1). Coupled with the poor actin retention and impaired actin kinetics observed at the population level, these observations imply that TarP plays a key role in coordinating actin kinetics at both the individual and population level. Together, these data emphasize the importance of the secreted effectors TarP and TmeA in generating a stable actin network with rapid recruitment and turnover kinetics. Additionally, our findings impress the importance of quantitatively analyzing individual invasion events to identify relevant characteristics that are masked in population-level analyses.

Arp2/3 recruitment dynamics at entry sites displays dependence on TmeA and TarP

One explanation for impaired actin kinetics following TarP and TmeA deletion is that loss of either effector prevents optimal recruitment of the actin nucleator Arp2/3. Indeed, a recent study suggested that the respective signal transduction capabilities of TarP and TmeA form a functional collaboration that enhances recruitment of Arp2/3 and increases the rate of actin polymerization [23,24]. As such, we monitored the recruitment of fluorescent Arp3 at the entry sites of wild-type, *cis*-complemented, or effector deletion strains in order to assess the contribution of effector collaboration on Arp2/3 recruitment during invasion (Fig. 2). We observed recruitment of Arp3 in all strains except for Δ TmeA/ Δ TarP, indicating that Arp2/3 recruitment is contingent upon the presence of either TarP or TmeA (Fig. 2A,B). We noted that *cis*-TmeA EBs required an additional 20 seconds to recruit Arp3 at a comparable intensity to either wild-type or *cis*-TarP strains (Fig. 2B), which is consistent with the slight delay in peak actin intensity observed prior (Fig. 1B). Surprisingly, TmeA deletion did not substantially alter the intensity of Arp3 recruitment, whereas TarP deletion resulted in dim and transient Arp3 recruitment (Fig. 2B). In conjunction with the absence of Arp3 recruitment in the Δ TmeA/ Δ TarP strain, these data suggest that both TarP and TmeA are independently capable of Arp3 recruitment. Nevertheless, the apparent differences between the Arp3 recruitment profiles of Δ TarP and Δ TmeA are inconsistent with a synergetic interaction between the effectors, instead indicating that Arp2/3 recruitment is primarily driven by TarP. However, we observed that Arp3 recruitment at the entry sites of Δ TmeA EBs was visibly more transient than Arp3 recruitment in either *cis*-TmeA or wild-type strains, indicating a potential role for TmeA in promoting stable recruitment of Arp2/3.

In order to clarify the respective contributions of TarP and TmeA toward Arp2/3 recruitment, we monitored the kinetics of Arp3 recruitment and turnover for each strain tested above. We noted that the recruitment kinetics of Arp3 were largely similar in all strains except for Δ TarP, which recruited Arp3 nearly 4-fold slower than wild-type or *cis*-complemented strains (Wild-type=0.079 fold/sec, Δ TarP=0.020 fold/sec) (Fig. 2C, Table S2). Consistent with the delay in peak Arp3 intensity, *cis*-TmeA EBs possessed a

slightly lower median Arp3 recruitment rate (*cis*-TmeA=0.059 fold/sec) than wild-type or *cis*-TarP EBs, although the distribution of rates is comparable among all three strains. Finally, TmeA deletion did not alter the recruitment kinetics of Arp3, as Δ TmeA EBs recruited Arp3 at a rate comparable to wild-type or *cis*-TarP EBs (Δ TmeA= 0.091 fold/sec, *cis*-TarP= 0.095 fold/sec). Altogether, these data indicate that while TmeA is independently capable of recruiting Arp2/3, it does so at a far slower rate than strains which contain TarP. As such, we conclude that the presence of TarP is a key determinant of rapid Arp3 recruitment.

During imaging, we noted that Arp3 recruitment by Δ TmeA EBs was visibly more transient than *cis*-TmeA or wild-type EBs, which may indicate that TmeA signaling is necessary for optimal retention of Arp2/3. In agreement with this, we found that the turnover rate of Arp3 was substantially higher at Δ TmeA entry sites (Δ TmeA=0.091 fold/sec) than wild-type (0.035 fold/sec) or *cis*-TmeA (0.054 fold/sec) (Fig. 2D). Furthermore, pairwise correlation analyses revealed that the Arp3 turnover rate of Δ TmeA EBs was positively correlated with both its corresponding recruitment rate and its recruitment intensity, together implying that rapid and intense Arp3 recruitment results in proportionally rapid Arp3 turnover (Table 2). Collectively, this demonstrates that TmeA signaling contributes to the stability of Arp2/3 following its recruitment, such that TmeA deletion results in poor retention of Arp2/3, attenuated actin kinetics, and inefficient engulfment of *Chlamydia*. Meanwhile, TarP deletion resulted in a drastic attenuation of Arp3 turnover kinetics relative to wild-type or *cis*-TarP strains (*cis*-TarP=0.070 fold/sec, Δ TarP=0.023 fold/sec), indicating that loss of TarP signaling dramatically alters both recruitment and turnover of Arp2/3. In summary, these data indicate that TarP and TmeA signaling are mutually important for optimal Arp2/3 kinetics, each regulating distinct attributes of Arp2/3 recruitment. TarP signaling is associated with robust Arp2/3 recruitment, while TmeA signaling is required to maintain Arp2/3 stability within entry sites. Coupled with the attenuated actin kinetics and poor invasion efficiency of Δ TarP and Δ TmeA strains (Fig.

1), we conclude that efficient invasion is reliant upon the co-regulation of Arp2/3 kinetics by TarP and TmeA.

Table 2: Pairwise correlation analyses of Arp3 kinetics following deletion or *cis*-complementation of TarP and TmeA

	Recruitment Rate vs. Turnover Rate	Recruitment Rate vs. Intensity	Turnover Rate vs. Intensity	Turnover Rate vs. Duration
Arp3 <i>cis</i> -TmeA	r= 0.004	r= 0.657	r= 0.294	r= 0.224
Arp3 Δ TmeA	r= 0.549	r= 0.377	r= 0.775	r= 0.447
Arp3 <i>cis</i> -TarP	r= 0.003	r= 0.319	r= 0.304	r= 0.269
Arp3 Δ TarP	r= 0.612	r= 0.519	r= 0.452	r= 0.259

Negligible	Weak	Moderate	Strong	Very Strong
0.00 - 0.19	0.20 - 0.39	0.40 - 0.59	0.60 - 0.79	0.8+

Formin 1 is recruited during invasion in a TarP-dependent and TmeA-independent manner

Despite the mutual importance of TarP and TmeA toward efficient invasion, we noted that the recruitment profiles of both actin and Arp3 differed substantially between Δ TarP and Δ TmeA strains. In particular, we noted that the kinetics of actin and Arp3 were more substantially attenuated during Δ TarP invasion than Δ TmeA invasion. One possible explanation for this difference may be the presence of additional TarP-specific factors which influence the kinetics of Arp2/3 and actin. Arp2/3 is known to collaborate with a class of actin nucleators known as formins, which generate elongated actin filaments by substantially increasing the polymerization rate of actin. These elongated filaments are bound by Arp2/3, generating branched actin filaments that may be acted upon by formins, consequently increasing the kinetics of both Arp2/3 and actin recruitment. As such, we tested whether formins are recruited during invasion, and whether their recruitment was contingent upon the presence of TarP or TmeA. We observed the robust recruitment of Formin 1 (Fmn1) at the entry sites of wild-type, *cis*-complemented, and Δ TmeA strains, which was absent in Δ TarP and Δ TmeA / Δ TarP strains, suggesting that Fmn1 recruitment is TarP-

dependent (Fig. 3A,B). TmeA deletion did not appreciably alter the recruitment intensity of Fmn1 relative to either *cis*-TmeA or wild-type *Chlamydia* (wild-type=10.7 ± 1.7 fold, *cis*-TmeA=9.9 ± 1.4 fold, Δ TmeA=8.5 ± 0.6 fold), indicating that recruitment of Fmn1 is mediated primarily by TarP and largely independent of TmeA. Additionally, we observed that Fmn1 recruitment was often persistent among wild-type and *cis*-complemented strains, resulting in a protracted turnover phase wherein Fmn1 is retained for 300+ seconds after peak intensity (Fig. 3B). This phenomenon was absent during Δ TmeA invasion; rather, Fmn1 recruitment in this strain rapidly achieved peak intensity before equally rapidly returning to basal intensity. Finally, Δ TarP and Δ TmeA / Δ TarP EBs failed to recruit Fmn1, yielding only trace RFP signals at sites where these strains contact host cells. Collectively, these data indicate that Fmn1 is recruited during invasion in a TarP-dependent manner, while TmeA signaling appears to influence the retention of Fmn1 at invasion sites, akin to its proposed role in supporting Arp2/3 stability.

To further examine the influence of TarP and TmeA signaling on Fmn1 recruitment, we compared the kinetics of Fmn1 recruitment between strains (Fig. 3C, Table S3). Once again, we observed that *cis*-TmeA EBs exhibited a marginally slower Fmn1 recruitment rate (*cis*-TmeA=0.077 fold/sec) than either wild-type (0.106 fold/sec) or *cis*-TarP (0.127 fold/sec) EBs, which mirror our findings regarding the kinetics of actin and Arp3. Thus, although *cis*-TmeA recruitment kinetics are generally slightly reduced relative to wild-type *Chlamydia*, the pattern of deviation is consistent between actin, Arp3, and Fmn1, indicating that the recruitment of these proteins is co-regulated. Fmn1 recruitment kinetics were not altered by TmeA deletion (Δ TmeA=0.092 fold/sec) but were reduced by over 10-fold following TarP deletion (Δ TarP=0.013 fold/sec). Together, this implies that TarP is the primary determinant of Fmn1 and Arp2/3 recruitment kinetics.

Interestingly, we noted that Δ TmeA EBs achieved a significantly higher Fmn1 turnover rate than wild-type *Chlamydia* (Wild-type=0.041 fold/sec, Δ TmeA=0.070 fold/sec), which is reminiscent of the pattern observed during Arp3 turnover (Fig. 3D). Indeed, correlation analyses indicated that, similar to

Arp3, the turnover rate of Fmn1 among Δ TmeA EBs was correlated with its corresponding recruitment rate and recruitment intensity (Table 3), suggesting that Fmn1 evacuates Δ TmeA entry sites at a rate proportional to its recruitment. Thus, although TmeA signaling has a negligible impact on the recruitment kinetics of Fmn1 and Arp2/3, it is essential for stable association of these actin nucleators within *Chlamydia* entry sites. In contrast, TarP deletion prevented the recruitment of Fmn1, resulting in a nearly negligible Fmn1 turnover rate (Δ TarP=0.009 fold/sec). Collectively, these data reveal that the actin nucleators Arp2/3 and Fmn1 are robustly recruited during invasion to establish an intense and highly localized actin network with rapid kinetics. TarP secretion is essential for Fmn1 recruitment, and is required for optimal Arp2/3 recruitment, indicating that TarP is a key regulator of actin nucleator recruitment. In contrast, TmeA signaling was revealed to promote stable retention of actin nucleators within *Chlamydia* entry sites, which was crucial for efficient pathogen engulfment. Thus, despite having only a limited effect on the recruitment kinetics of actin and its nucleators, TmeA signaling may be necessary for optimal activation, and thus retention of Arp2/3. A failure to activate and integrate Arp2/3 within actin networks would limit the number of binding sites for Fmn1, effectively promoting its turnover and restricting the influence of Fmn1 and Arp2/3 on actin remodeling.

Table 3: Pairwise correlation analyses of Fmn1 kinetics following deletion or *cis*-complementation of TarP and TmeA

	Recruitment Rate vs. Turnover Rate	Recruitment Rate vs. Intensity	Turnover Rate vs. Intensity	Turnover Rate vs. Duration
Fmn1 <i>cis</i> -TmeA	r= 0.516	r= 0.654	r= 0.250	r= 0.088
Fmn1 Δ TmeA	r= 0.647	r= 0.196	r= 0.313	r= 0.498
Fmn1 <i>cis</i> -TarP	r= 0.333	r= -0.02	r= 0.28	r= 0.208
Fmn1 Δ TarP	r= 0.935	r= 0.658	r= 0.7	r= 0.592

Negligible	Weak	Moderate	Strong	Very Strong
0.00 - 0.19	0.20 - 0.39	0.40 - 0.59	0.60 - 0.79	0.8+

Inhibition of filamentous and branched nucleation of actin at the sites of entry reduces invasion efficiency and significantly alters actin recruitment kinetics

As a consequence of actin remodeling, *Chlamydia* reconfigures host membranes into invasion-associated structures such as hypertrophic microvilli, which assist in the capture and engulfment of invading bacteria. These structures are associated with the actin elongating properties of formins such as Fmn1, which is recruited during invasion in a TarP-dependent manner (Fig. 3). This finding confirms an earlier observation from a previously published RNA interference screen in *Drosophila* S2 cells that identified formin and Diaphanous as being required for *Chlamydia* infection. In light of these observations, we transfected Cos7 cells with GFP-fusion constructs of the mammalian Diaphanous homologs mDia1 (mEmerald-mDia1) and mDia2 (mEmerald-mDia2) and tracked their recruitment around invading *Chlamydiae* (Fig. 4A). We identified recruitment of Fmn1, mDia1, and mDia2 at wild-type *Chlamydia* entry sites, indicating that multiple species of formin are recruited during invasion. We utilized the pan-formin inhibitor SMIFH2 to pharmacologically inhibit formin activity in order to circumvent the observed cytotoxicity associated with simultaneous knockdown of Fmn1, mDia1, and mDia2. SMIFH2 is ideally suited for this purpose, since it inhibits all three formin species by blockade of their conserved formin-homology 2 (FH2) domains, thus preventing formin-mediated actin polymerization [43]. Importantly, we utilized SMIFH2 at a relatively low concentration (10 μ M), which is approximately 4-fold lower than the effective concentration to inhibit myosin superfamily proteins (Fig. S5) [44]. Additionally, we employed the Arp2/3 inhibitor CK666, which prevents Arp2/3 branching activity by occluding the actin binding cleft between subunits Arp2 and Arp3 [45]. The Arp2/3 complex is known to be required for efficient invasion [9], and thus serves as a basis for comparison regarding the potential effects of formin inhibition .

First, we investigated whether the activities of formin and Arp2/3 are necessary for invasion by comparing the efficiency of *Chlamydia* invasion in the presence and absence of SMIFH2, CK666, or both (Fig. 4B). Cos7 cells were pre-treated with inhibitors for 60 min prior to inoculation with *C. trachomatis*

serovar L2 EBs. We normalized our data to the efficiency of invasion in absence of inhibitor in order to directly compare the effects of formin and/or Arp2/3 inhibition on invasion. Inhibiting the activity of either formin or Arp2/3 yielded a comparable impediment to invasion, each reducing invasion efficiency by roughly 40%, while simultaneous inhibition of formin and Arp2/3 reduced invasion efficiency by 60%. Together, these data suggest that while formin and Arp2/3 activity are independently important for invasion, their combined inactivation causes a more substantial impediment to pathogen entry.

To determine if this impairment in *Chlamydia* internalization is due to a deficiency in actin polymerization, we quantified the recruitment of GFP-actin at the site of invasion in the presence or absence of these inhibitors by time-lapse imaging of live Cos7 cells (Fig. 4C). *Chlamydia* robustly recruited actin in absence of inhibitor at an intensity of nearly 20-fold above background, which was reduced by over 10-fold following application of SMIFH2 or CK666. Interestingly, dual inhibition of formin and Arp2/3 did not further reduce actin recruitment but yielded a form of actin recruitment that was substantially slower and much more persistent, often failing to turn over throughout the duration of the experiment (Video S2). Altogether, these data indicate that the activities of formin and Arp2/3 are manipulated by *Chlamydia* to enhance actin remodeling, and that inhibition of either nucleator yields a deficiency in pathogen entry.

Recruitment and turnover of actin are slowed by inhibition of filamentous and branched actin nucleators

Earlier, we noted that mutant *Chlamydia* strains which failed to recruit and retain Fmn1 and/or Arp2/3 suffered defects to both actin kinetics and invasion efficiency, suggesting that stable association of both nucleators is necessary for optimal actin kinetics. Our data indicate that inhibition of either Arp2/3 or formin induces a comparable defect to both actin recruitment intensity and invasion efficiency, intimating that their respective actin branching and elongating properties are equally important for robust actin

kinetics. To test this claim, we compared the kinetics of actin recruitment and turnover at the invasion sites of cells treated with inhibitors for Arp2/3 and/or formin (Fig. 4D,E, Fig. S6). Inhibitor treatment reduced the rate of recruitment by 3-4 fold compared to mock-treatment (Mock=0.285 fold/sec, SMIFH2=0.082 fold/sec, CK666=0.072 fold/sec) (Fig. 4D) and altered the recruitment profile across multiple parameters, ultimately resulting in a narrower range of recruitment rates (Table S4). Together, this indicates that when only one nucleator is operational, the rate of actin recruitment is slower and more consistent. Furthermore, co-inhibition of formin and actin yielded an even more narrow range of actin recruitment rates (Range CK+SMI=0.044) and reduced the overall rate by 10-fold (CK+SMI=0.015 fold/sec). Since actin recruitment was not completely ablated by dual-inhibition of formin and Arp2/3, these data imply the presence of a formin- and Arp2/3-independent mechanism of actin recruitment at the site of invasion. However, the resultant actin network polymerizes very slowly and more uniformly than the actin network created by host actin nucleators. Collectively, this suggests that formin and Arp2/3 activity contributes to efficient invasion by enhancing the recruitment kinetics of actin, resulting in the rapid establishment of a robust actin network at the site of invasion.

Interestingly, the turnover phase of actin kinetics shares many features in common with the recruitment phase (Fig. 4E). Inhibition of formin or Arp2/3 reduced the median turnover rate by 3-4 fold compared to mock treatment (Mock=0.096 fold/sec, SMIFH2=0.026 fold/sec, CK666=0.031 fold/sec) (Fig. 4E, Table S4), which is comparable to the reduction observed in the recruitment phase of actin. Moreover, inhibition of formin or Arp2/3 also altered the turnover profile of actin, generating a narrower range of turnover rates (Table S4). Given that recruitment and turnover are comparably altered by inhibition of formin or Arp2/3, our data imply a link between these phases such that rapid/slowed recruitment is paired with rapid/slowed turnover. This is also true for when formin and Arp2/3 are simultaneously inhibited, which reduces the turnover rate of actin by over 10-fold (CK+SMI=0.005 fold/sec) compared to mock treatment. To confirm that comparisons derived from the violin plots are reflective of the overall kinetics

for each recruitment condition, we performed a linear regression analysis on the mean rate of recruitment and turnover for each phase (Fig. S6). We noted that the trends observed in violin plots (Fig. 4D,E) were also observed in the averaged rate slopes for all conditions tested (Fig. S6A,B), indicating that differences between individual rate distributions are reflective of the overall kinetics for each condition. In sum, our data indicate that inhibition of host actin nucleators substantially impairs rapid actin recruitment and turnover at the site of invasion, while only having a minor effect on the slow, lingering phase of actin turnover.

Finally, we wanted to evaluate whether the trends observed in actin recruitment and turnover at the population level were preserved at the single EB level (Table 4). Consistent with the correlation analyses conducted on *cis*-TarP and *cis*-TmeA, we observed that mock-treated wild-type *Chlamydia* exhibited negligible correlation between the rate of actin recruitment and turnover. In contrast, inhibition of either formin or Arp2/3 altered this relationship such that a moderate correlation existed between recruitment and turnover rates. Interestingly, we observed that the rate of actin recruitment and turnover exhibited a moderate correlation following inhibition of Arp2/3, and negligible correlation upon inhibition of formin. Collectively, these data suggest that formin and Arp2/3 uniquely contribute to actin dynamics at entry sites, such that inhibition of either nucleator produces divergent effects on the regulation of actin networks among individual EBs. Notwithstanding, we conclude that optimal kinetics of actin recruitment are achieved when both formin and Arp2/3 are active, suggesting that *Chlamydia* relies on the nucleation of both branched and filamentous actin for optimal actin remodeling.

Table 4: Pairwise correlation analyses of actin kinetics following inhibition of formin and Arp2/3

	Recruitment Rate vs. Turnover Rate	Recruitment Rate vs. Intensity	Turnover Rate vs. Intensity	Turnover Duration vs. Intensity
Actin Untreated	r= 0.173	r= 0.371	r= 0.269	r= 0.043
Actin SMIFH2	r= 0.581	r= 0.084	r= -0.084	r= 0.382
Actin CK666	r= 0.428	r= 0.477	r= 0.472	r= 0.196
Actin CK+SMI	r= -0.003	r= -0.059	r= 0.334	r= 0.098

Negligible	Weak	Moderate	Strong	Very Strong
0.00 - 0.19	0.20 - 0.39	0.40 - 0.59	0.60 - 0.79	0.8+

Formin and Arp2/3 are co-recruited to the site of invasion

The observation that formin and Arp2/3 promote actin recruitment during invasion raise the possibility that these nucleators act collaboratively, thereby enhancing the capacity of *Chlamydia* to generate actin-rich invasion structures. If nucleator collaboration does indeed play a role in invasion, we expect that both formin and Arp2/3 will be present within a single invasion event, and that inhibiting either nucleator will impair recruitment of its collaboration partner. Although multiple species of formin were recruited during invasion (Fig. 4A), we chose to monitor Fmn1 since this protein was specifically identified in a prior siRNA screen of cytoskeletal effectors with a role in *Chlamydia* invasion [46]. We observed that GFP-Fmn1 was robustly recruited during invasion and experienced a substantial defect in recruitment upon pharmacological inhibition of Arp2/3 (Fig. 5A). Likewise, GFP-Arp3, which has been validated as a marker for the Arp2/3 complex [47], was robustly recruited during invasion and also demonstrated reduced recruitment following inhibition of formin (Fig. 5B). Altogether, this indicates that recruitment of Fmn1 and Arp2/3 are reciprocally enhanced by their respective actin nucleating functions and subsequent actin polymerization.

Next, we determined whether Fmn1 and Arp2/3 are co-recruited within individual invasion events, thus providing the necessary context for nucleator collaboration. To do this, we co-expressed GFP-Fmn1 and mCherry-Arp3 and measured the recruitment of both proteins during invasion by time-lapse imaging followed by fluorescence quantification (Fig. 5C). We observed that all events we monitored

exhibited both Fmn1 and Arp3 accumulation, indicating that they are co-recruited by *Chlamydia*. To measure the timing of their arrival, we normalized the intensity of recruitment to the maximal MFI of each protein at each timepoint, expecting that any delays in protein recruitment would be represented by a shift in their recruitment profile. In contrast, we noted that the recruitment profiles of both Fmn1 and Arp3 were remarkably similar and achieved maximal MFI at the same timepoint, indicating that both proteins arrived at the entry site within 20 seconds of each other, the temporal resolution of our imaging protocol. Given both the simultaneous recruitment of Fmn1 and Arp2/3 and the reciprocal dependence of their respective activities for optimal recruitment, we conclude that Fmn1 and Arp2/3 collaborate during invasion to promote actin recruitment. This observation is particularly novel, given that there are no other instances of the utilization of Fmn1 by bacterial pathogens. Lastly, given the robust recruitment of Fmn1, we were interested in whether other formin species such as DRFs, which require upstream activation by Rho-GTPases, could also participate in nucleator collaboration. To this end, we found that mEmerald-mDia1 and mEmerald-mDia2 were also recruited during invasion, and that their recruitment was impaired by Arp2/3 inhibition (Fig. 5D), suggesting that both DRF and non-DRF formins collaborate with Arp2/3 during invasion.

Recruitment and turnover profiles of Fmn1 and Arp2/3 are altered by reciprocal inhibition of their respective actin nucleation function

Using the same methodology to evaluate actin kinetics (Fig. 1D,E), we compared the recruitment and turnover profiles of Fmn1 and Arp2/3 to more closely evaluate how inhibition of each protein alters their recruitment and turnover dynamics during invasion. Compared to mock treatment, inhibition of Fmn1 decreased the rate of Arp3 recruitment by 34 percent (Mock=0.079 fold/sec, SMIFH2=0.052 fold/sec), while inhibition of Arp2/3 reduced the rate of Fmn1 recruitment by 47 percent (Mock=0.106 fold/sec, CK666=0.056 fold/sec) (Fig. 5E, Table S5). Together, this indicates that collaboration between the activities

of Fmn1 and Arp3 not only enhance the rate of actin recruitment (Fig. 4D), but also reciprocally enhance their respective recruitment rates. As such, we conclude that Arp2/3-mediated actin branching produces additional barbed-ends for Fmn1 binding; reciprocally, Fmn1 activity produces elongated actin filaments which allow efficient incorporation of Arp2/3 into actin networks generated by invading *Chlamydiae*.

Shifting our focus onto turnover, we found that CK666 treatment reduced the turnover rate of Fmn1 by 4-fold compared to mock-treatment (Mock=0.041 fold/sec vs. CK666=0.009 fold/sec), while SMIFH2 treatment did not significantly alter the rate of Arp3 turnover (Mock=0.019 fold/sec vs. SMIFH2=0.024 fold/sec) (Fig. 5F, Table S5). Furthermore, we found that the turnover rate of Fmn1 among individual EBs was moderately correlated with its corresponding peak intensity ($r=0.402$), which was not observed upon inhibition of Arp2/3 ($r=0.074$) (Table 5). This discrepancy may be due to a relative lack of Fmn1 binding sites coupled with poor actin turnover following inhibition of Arp2/3 (Fig. 4E), such that the turnover rate of Fmn1 is dictated by binding site availability in inhibitor-treated cells. In contrast, rapid turnover of Fmn1 in mock-treated cells is potentially due to the relative abundance of Fmn1 binding sites coupled with rapid actin turnover, such that Fmn1 turnover is likely dictated by actin turnover dynamics. Meanwhile, we noted that SMIFH2 treatment did not alter the turnover rate of Arp2/3 (Fig. 5F), nor did it exert any observable effect on the regulation of Arp2/3 turnover as indicated by pairwise correlation analysis (Table 5). Thus, we conclude that turnover of Fmn1 and Arp2/3 are governed by different mechanisms, wherein Arp2/3 turnover occurs via a process that is independent of Fmn1 activity.

Table 5: Pairwise correlation analyses of Fmn1 and Arp3 kinetics in presence and absence of inhibitor.

	Recruitment Rate vs. Turnover Rate	Recruitment Rate vs. Intensity	Turnover Rate vs. Intensity	Turnover Duration vs. Intensity
Fmn1 Untreated	r= 0.367	r= 0.443	r= 0.398	r= 0.087
Fmn1 CK666	r= 0.119	r= 0.248	r= 0.074	r= 0.268
Arp3 Untreated	r= 0.395	r= 0.788	r= 0.596	r= 0.367
Arp3 SMIFH2	r= 0.487	r= 0.567	r= 0.582	r= 0.101
Negligible	Weak	Moderate	Strong	Very Strong
0.00 - 0.19	0.20 - 0.39	0.40 - 0.59	0.60 - 0.79	0.8+

Overall, this study highlights the remarkable complexity of the interaction between invading *Chlamydia* and the host cytoskeleton, identifying several novel interactions which culminate to support rapid actin kinetics and efficient pathogen engulfment. In play are at least two effectors (TarP and TmeA), signaling from accessory cell surface receptors, and the existing cross-regulatory machinery of actin nucleation, polymerization, and turnover (Fig. 6). Adding to this complexity is the various functional interactions between multiple formins/formin-like proteins with Arp2/3. The recruitment and turnover kinetics of mDia1 and mDia2 were similarly sensitive to CK666-mediated inhibition of the Arp2/3 complex (Fig. S7, Table S6,7), suggesting that multiple formin species may participate in nucleator collaboration. Thus, our data indicate that filamentous and branched actin nucleation and polymerization constitute a fundamental interaction in which formins in general collaborate with Arp2/3 during invasion following their recruitment by TarP and TmeA.

Discussion:

In this study, we demonstrated that co-regulation of Arp2/3 kinetics by the *Chlamydia* effectors TarP and TmeA, coupled with TarP-dependent recruitment of Fmn1, led to the establishment of a robust actin modulatory network at pathogen entry sites to promote internalization. By monitoring the kinetics of recruitment, we identified a functional collaboration between the actin nucleating and polymerizing activities of formin and Arp2/3, which accelerated the rate of actin recruitment as well as reciprocally enhancing the recruitment of actin nucleators. Furthermore, we emphasized the necessity for actin turnover in order to achieve pathogen internalization and demonstrated that signaling from both TarP and TmeA were essential for rapid actin turnover and optimal pathogen internalization.

Actin dynamics at the site of chlamydial invasion

Intracellular pathogens often target the actin cytoskeleton during entry, leading to robust actin recruitment and subsequent formation of invasion-associated structures. Although *Chlamydia* has been demonstrated to use a plurality of entry mechanisms to achieve internalization (filopodial capture, clathrin-mediated endocytosis, macropinocytosis, membrane ruffling), we were unable to distinguish the actin dynamics associated with each mechanism. Therefore, while our study impressed the importance of TarP and TmeA signaling toward optimal actin kinetics, we were unable to identify whether deletion of either effector altered which entry mechanisms were utilized by invading *Chlamydiae*. Likewise, further study is required to determine the role of formin and Arp2/3 toward the assembly of each invasion-associated structure, and whether inhibition of either actin nucleator prevents the utilization of a given entry mechanism.

Relative to *Chlamydia*, actin recruitment in other intracellular bacteria is longer lived [17,48], lasting on the order of 5-15 minutes; in contrast, we have shown that *Chlamydia* actin recruitment is both

robust and transient, emphasizing the importance of rapid actin accumulation and turnover during its invasion. To our knowledge, no equivalent study on the rate of actin recruitment during the internalization of an intracellular pathogen has been conducted, thus a direct comparison on the kinetics of actin recruitment between pathogens is not possible. As such, we believe our study provides a valuable new approach that could be used in future studies to improve contextualization of protein dynamics for other intracellular pathogens in addition to *Chlamydia*.

Using these tools, we found that secretion of the *Chlamydia* effectors TarP and TmeA led to rapid recruitment of actin, which was due in part to the collaboration between Fmn1 and Arp2/3. One explanation for the high rate of recruitment observed in our study is the establishment of a positive feedback loop, where actin branching by Arp2/3 and filament elongation by Fmn1 create additional nodes for Arp2/3 and Fmn1 to bind (Fig. 6). Iterations of this process would account for both the high recruitment rate of actin in addition to providing an explanation for why inhibition of Fmn1 and Arp2/3 comparably affect the recruitment rate of actin. However, this model does not immediately make apparent why actin recruitment is transient, nor does it provide explanation for the slow and persistent actin recruitment observed following inhibition of both formin and Arp2/3. Other host actin nucleators such as SPIRE, APC, or Cordon-bleu have not yet been identified in siRNA screens of invasion-associated proteins, nor has any evidence of their involvement in *Chlamydia* invasion been given [49]. Although TarP has been shown via pyrene assay to serve as an actin nucleator, it remains unknown whether it also performs this function in cell lines or *in-vivo* [50]. However, based on results described here, TarP deletion drastically impaired the kinetics of recruitment and turnover of both actin and its nucleators, indicating that actin nucleation is predominantly driven by host nucleators rather than TarP itself.

In addition to rapid actin recruitment, we also observed a two-phase actin turnover process starting with a brief fast turnover phase (100-180 sec) followed by a prolonged slow turnover phase (400-600 sec). The rate of actin turnover was reduced by deletion of either TmeA or TarP, or by inhibition of

either formin or Arp2/3, collectively indicating that rapid actin turnover is an effector-driven process which is contingent upon the activity of actin nucleators. Although this process is relatively understudied in intracellular bacterial invasion, actin turnover is of clear importance for bacterial internalization, as dysregulation of actin turnover machinery commonly impairs bacterial entry. For instance, impairment or hyperactivation of the actin depolymerizing protein ADF-cofilin restricts internalization of *S. enterica*, while overexpression of the ADF-cofilin kinase LIMK reduced *L. monocytogenes* ruffle formation and internalization [51,52]. In *C. burnetii* and *L. monocytogenes*, time-course experiments tracking actin recruitment also demonstrated a clear spike in actin recruitment followed by a two-phase rapid/residual turnover profile [48,52]. Thus, the actin turnover profile observed in our study may simply reflect normal actin turnover dynamics, as opposed to being intrinsic to invading *Chlamydia*. Given the importance of host actin depolymerization factors like gelsolin, ADF-cofilin and their regulators for efficient internalization of other intracellular bacteria, it may be that such proteins are also responsible for actin turnover during *Chlamydia* invasion [40,53–55]. Indeed, an siRNA screen of *C. muridarum* invasion-associated proteins identified four actin depolymerization factors, ADF-cofilin, cyclase associated protein (CAP), Coronin, and β -thymosin, each of which reduced bacterial entry upon silencing (Fig. 6) [46]. Thus, one avenue for future study is to characterize the role of ADF-cofilin and other actin depolymerization factors in *Chlamydia* invasion and whether dysregulation or silencing of these proteins alters the kinetics of actin turnover. Furthermore, additional study is required to determine what circumstances prompt the termination of actin recruitment and how *Chlamydia* achieves actin recruitment in absence of host nucleators. Of particular interest is the apparent correlation between actin turnover and the presence of functional TarP, TmeA, Arp2/3, and Formin 1. This raises a number of possible mechanisms that trigger rapid turnover, including actin network density, physical stress that might sensitize the actin network to turnover, or the actin network having a configuration that is preferred by the local depolymerization machinery present.

Divergent effects of TarP and TmeA signaling on nucleator recruitment

Although we observed that both TarP and TmeA were independently capable of recruiting Arp2/3, we noted that the predominant fraction of Arp2/3 recruitment was linked to TarP (Fig. 2C), while TmeA signaling was associated with Arp2/3 retention within entry sites. Likewise, TarP deletion prevented recruitment of Fmn1 and yielded a greater impediment to actin kinetics than TmeA deletion (Fig. 1B-E, 3B). Thus, our data indicate that TarP signaling is essential for nucleator collaboration, which likely occurs independent of TmeA signaling. Nevertheless, our data impress the importance of TmeA signaling in promoting the retention of actin nucleators and enhancing the efficiency of actin remodeling. It is unclear if Arp2/3 collaborates with Fmn1 in the context of TmeA-dependent, but TarP-independent invasion and actin dynamics, however available data indicate no collaboration. It may be that the actin network formed by the TmeA/N-WASP/Arp2/3 axis does not offer binding sites for Fmn1, preventing any collaboration from occurring. Nevertheless, our data corroborate recent findings obtained via *in vitro* pyrene-actin assays which indicate that TmeA signaling through N-WASP leads to Arp2/3 activation. Within the context of our model, an intriguing possibility is that TmeA signaling maintains an active pool of N-WASP to activate Arp2/3 complexes bound along the length of actin filaments. However, this is speculative given that we did not observe N-WASP recruitment in prior live-cell imaging experiments that lasted for 360 s [9]. An added complexity is the potential involvement of growth factor receptors, e.g. EGFR, FGFR, integrins, etc., which could provide the signals to activate Arp2/3, and thus would be redundant to TarP and TmeA [56–60]. Besides a potential compensatory role for growth factor receptors, an additional layer of complexity could come from signaling cross-talk. Given the plurality of factors involved in *Chlamydia* invasion, much more research is needed to identify their individual contributions and integrate them into a more comprehensive understanding of which signaling pathways influence collaboration between branched and filamentous actin nucleators.

Although TarP signaling has not yet been definitively linked to the activation of DRFs, we identified the recruitment of two DRFs, mDia1 and mDia2, which require activation by Rho-GTPases prior to serving as actin nucleators or filament elongation factors. Since TarP signals through Rac1 [9,22], which is known to activate mDia1 and mDia2 [61,62], our data support a model where TarP signaling contributes to the assembly of both branched and filamentous actin (Figs. 2,3). Interestingly, Fmn1 recruitment was found to be TarP-dependent despite the fact that Fmn1 activity, unlike DRFs, is not contingent upon activation by Rho-GTPases. Fmn1 recruitment is likely mediated by the actin network nucleated by Arp2/3, with the barbed ends of each branch serving as binding sites, and thus may be indirectly recruited as a product of effector-mediated actin remodeling. However, since Fmn1 recruitment was absent in TarP-deficient *Chlamydiae*, it seems that Arp2/3 which is activated by the alternate TmeA-dependent mechanism is incapable of compensating for the recruitment of Fmn1. Thus, the means by which Arp2/3 is activated appears to determine whether it will collaborate with Fmn1 during invasion. Nevertheless, the recruitment of multiple formin species highlights the importance of filamentous actin polymerization toward *Chlamydia* invasion, particularly since it is known that *Chlamydia* induces the assembly of hypertrophic microvilli, whose assembly is reliant upon the activity of formins [63–65]. Finally, since the pan-formin inhibitor SMIFH2 is expected to inhibit all three formin species identified in our study [43], we are incapable of decoupling the effects of Fmn1, mDia1, and mDia2 in nucleator collaboration and subsequent actin remodeling. Attempts to address the individual contributions of each formin species via RNA interference were thwarted by cytotoxicity upon silencing of these proteins, preventing the use of formin knockdown cells in downstream applications (i.e kinetics and invasion efficiency). Overall, our data demonstrate the importance of formins toward *Chlamydia* invasion and introduce the possibility that TarP signaling contributes more to actin remodeling than was previously appreciated.

Throughout this study, we obtained evidence that actin dynamics associated with *Chlamydia* invasion have core and auxiliary elements. This is based on the correlation between the recruitment and

turnover phases of actin dynamics. At the population level, actin dynamics, secretion of TarP and TmeA, or inhibition of either nucleators led to the reduction in the rates of recruitment and turnover. However, upon detailed analysis where the recruitment phase for individual elementary bodies were matched with their corresponding turnover phase, we could not demonstrate significant correlation. Elementary bodies exhibiting rapid recruitment gave rise to rapid and slow turnover equally, and vice versa. When viewed from the perspective of an invasion process involving core and auxiliary components, the variability at the single-EB level could be accounted for by the accessory factors, which could include local concentration of actin, barbed ends, depolymerizing factors, actin crosslinkers, capping, etc., while an intact core component would explain the generally consistent trends observed at the population level. At the outset of invasion, *Chlamydia* makes contact and subsequently activates an assortment of host receptors (EGFR, PDGFR, ER α / β , β 1-Integrin, etc.), which may influence the recruitment of actin, its nucleators, and actin depolymerization factors (Fig. 6) [46,66,67]. Furthermore, live-cell imaging of *Chlamydia* invasion did not reveal any consistent pattern of cell surface location of entry, instead occurring stochastically across the surface of the cell. As such, local variations in the levels of preassembled actin filaments or the concentration of signaling co-factors may exist. In addition, a myriad of other cellular machinery may be differentially accessible for exploitation by *Chlamydia*. What we observed, however, is that the loss of either TarP or TmeA had a substantial effect on the rate and distribution of actin kinetics, indicating that TarP and TmeA are core components of invasion that expand the repertoire of signaling events by coordinating the utilization of multiple accessory signaling proteins. Consequently, effector secretion results in the recruitment of a cohort of proteins which influence actin dynamics and contribute to efficient invasion of *C. trachomatis*, or vice versa where additional signaling events assist *Chlamydia* in mediating efficient pathogen entry. In this context, EGFR, PDGFR, ER α / β , β 1-Integrin and others could be accessory signaling molecules that on their own are not sufficient to support efficient invasion and rapid actin dynamics. This is further evidenced by the fact that deletion of either TarP or TmeA strongly

669 attenuated pathogen internalization, indicating that activation of one or more of the above receptors is
670 insufficient to compensate for loss of either effector. Instead, their functional utility only becomes
671 apparent upon secretion of TarP and/or TmeA.

672

Materials and Methods:

Cell and Bacterial Culture

Green monkey kidney fibroblast-like (Cos7) cells and cervical adenocarcinoma epithelial (HeLa) cells were cultured at 37°C with 5% atmospheric CO₂ in Dulbecco's Modified Eagle Medium (DMEM; Gibco, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10 µg/mL gentamicin, 2 mM L-glutamine, and 10% (v/v) filter-sterilized fetal bovine serum (FBS). HeLa and Cos7 cells were cultured for a maximum of 15 passages for all experiments. McCoy B mouse fibroblasts (originally from Dr. Harlan Caldwell, NIH/NIAID) were cultured under comparable conditions. *Chlamydia trachomatis* serovar L2 (434/Bu) was propagated in McCoy cells and EBs were purified using a Gastrografin density gradient as described previously [68].

Reagents

SMIFH2 (Tocris, Minneapolis, MN, USA) and CK666 (Sigma-Aldrich, St. Louis, MO, USA) were diluted upon receipt to 100mM stock concentration in DMSO, dispensed into single-use aliquots and stored at -20°C for no longer than 1 year after receipt. SMIFH2 was diluted to a working concentration of 10µM (1:10000) in supplemented DMEM, and CK666 was diluted to a working concentration of 100µM (1:1000) in supplemented DMEM.

Invasion Assay

Efficiency of *C. trachomatis* invasion in HeLa cells was performed as described previously [12]. Briefly, HeLa cells were seeded in 24-well plates containing acid-etched glass coverslips and allowed to adhere overnight. Cells were pretreated with SMIFH2 (10µM) (Tocris, Minneapolis, MN, USA), CK666 (100µM) (Sigma-Aldrich, St. Louis, MO, USA), or both, for 1 hour prior to infection. Following inhibitor treatment, cells were infected with EBs derived from wild-type *C. trachomatis* L2 (434/Bu), *C. trachomatis* in which

TarP, TmeA, or both were deleted by FRAEM (Δ TarP, Δ TmeA, Δ TmeA/ Δ TarP), or *C. trachomatis* in which TarP or TmeA expression was restored by *cis*-complementation (*cis*-TarP, *cis*-TmeA) at MOI=50. EBs were allowed to attach to HeLa cells for 30 min at 4°C. Cells were rinsed with cold HBSS before temperature shift to 37°C by the addition of pre-warmed DMEM + 10% FBS/2 mM L-glutamine and incubated at 37°C for 10 min. Cells were then washed with cold HBSS containing 100 µg/mL heparin to remove any transiently adherent EBs before fixation in 4% paraformaldehyde at room temperature for 15 min. Fixed cells were labeled with a mouse monoclonal anti-*Chlamydia* LPS antibody (BioRad CF 6J12, Hercules, CA, USA), rinsed with 1x PBS, and fixed once more in 4% paraformaldehyde for 10 min. Next, cells were permeabilized using 0.1% (w/v) Triton X-100 for 10 minutes at room temperature, rinsed with HBSS and labeled with rabbit polyclonal anti-*Chlamydia trachomatis* antibody (Abcam ab252762, Cambridge, MA, USA). Cells were then rinsed in 1x PBS and labeled with Alexa Fluor 488 anti-mouse (ThermoFisher #A28175, Waltham, MA, USA) and Alexa Fluor 594 anti-rabbit (ThermoFisher #A-11012) IgG secondary antibodies. Coverslips were mounted and observed on either a Leica DM500 (Leica, Allendale, NJ, USA) or Zeiss Axio Observer (Zeiss, Dublin, CA, USA) epifluorescence microscopes. Percent invasion efficiency was quantified as total EBs (red) – extracellular EBs (green)/total EBs (red) x 100%.

Quantitative live cell imaging of *Chlamydia* invasion

Cos7 cells were seeded onto Ibidi µ-Slide 8-well glass-bottomed chambers (Ibidi, Fitchburg, WI, USA) and allowed to adhere overnight prior to transfection. Cells were transfected with fluorescent proteins as indicated, using Lipofectamine 3000 (Thermo Fisher, Waltham, MA, USA) according to manufacturer directions. Transfection was allowed to proceed overnight before replacing media with fresh DMEM + 10% FBS/2 mM L-glutamine and allowing protein expression to continue for a total of 24 hours post-transfection. Transfection was verified on either a Leica SD6000 or Nikon CSU-W1 (Nikon, Melville, NY, USA) spinning disk confocal microscope prior to application of DMEM containing SMIFH2 (10µM), CK666

(100 μ M) or both. Wells were individually infected with CMTPX-labeled wild-type *C. trachomatis* L2 (434/Bu), unless otherwise indicated, at MOI=20 and promptly imaged using a 60x objective (NA 1.40) in a heated and humidified enclosure. Images were collected once every 20 seconds for 30 minutes, with focal plane maintained using an infrared auto-focusing system. Upon completion of the imaging protocol, the next well was infected and imaging repeated; mock-treated wells were imaged first to allow inhibitor treatment sufficient time to achieve inhibition. Images were compiled into videos using NIH ImageJ and analyzed to identify protein recruitment events. The mean fluorescence intensity (MFI) of such events was measured for each timepoint alongside the local background MFI of a concentric region immediately outside the recruitment event. Background MFI was subtracted from recruitment MFI for each timepoint and converted into fold recruitment by dividing the MFI of each timepoint against the baseline MFI, defined as the average MFI of five timepoints (100 sec) prior to the start of recruitment.

Plasmids and DNA preparation

pEGFP-Actin-C1 [69] was provided by Dr Scott Grieshaber (University of Idaho). pEGFP-N1-ACTR3 (Arp3-GFP) [47] was obtained from Dr Matthew Welch (Addgene plasmid #8462). Arp3-pmCherryC1 [70] was a gift from Christien Merrifield (Addgene plasmid #27682). mEmerald-mDia1-N-14 (Addgene plasmid #54157), mEmerald-mDia2-N-14 (Addgene plasmid #54159), and mRuby-LifeAct-7 (Addgene plasmid #54560) were gifted by Michael Davidson. pEGFPC2-FmnIso1b [71] was a gift from Philip Leder (Addgene plasmid #19320). pmCherryC1-FmnIso1b was generated by a variant of Gibson assembly termed FastCloning [72] and included removal of EGFP from pEGFPC2-FmnIso1b and insertion of mCherry derived from Arp3-pmCherryC1. Kanamycin-resistant transformants were selected and propagated for plasmid isolation prior to sequence verification using an mCherry-Fwd sequencing primer supplied by Eurofins Genomics. All plasmids were isolated using MiniPrep DNA isolation kits (Qiagen, Valencia, CA, USA) following a variant protocol for DNA isolation termed MiraPrep [73]. Following plasmid isolation, the

eluate was precipitated by addition of 3M sodium acetate (Invitrogen, Waltham, MA, USA) at 10% (v/v) of eluate volume followed by addition of 250% (v/v) absolute ethanol calculated after addition of sodium acetate. The mixture was incubated at 4°C overnight and centrifuged at 14,000×g for 15 minutes at 4°C. Supernatant was removed and 70% ethanol was added, followed by centrifugation at 14,000×g for 10 minutes at 4°C. Supernatant was removed once more, and precipitated DNA was resuspended in nuclease-free H₂O. Primers for assembly of pmCherryC1-FmnIso1b are as follows: (pEGFPC2) Fwd: 5' GGCGGAAGCGGAAGCATGGAAGGCACTCACTGCACC 3', Rev: 5' GCCCTTGCTCACCAT GGTGGCGACCGGTAGCG 3'; (pmCherryC1) Fwd: 5' CTACCGGTCGCCACCATGGTGAGCAAGGGCGAGG 3', Rev: 5' GTGAGTGCCTCCATGCTTCCGCTCCGCCG 3'.

Graphs and statistical analysis

Violin plots were made using the ggplot2 base package (version 3.1.0) as a component of the Tidyverse package (<https://cran.r-project.org/web/packages/tidyverse/index.html>) in rStudio (version 4.0.3). Wilcoxon ranked-sum tests to determine statistical significance between violin plots and tabulated statistical analyses of violin plots were generated using base R statistics and the moments package (version 0.14, <https://cran.r-project.org/web/packages/moments/index.html>) in rStudio and assembled in Excel (Microsoft, Redmond, WA, USA). Linear regression analyses, recruitment plots, invasion assays, and all statistics associated with these data (pairwise T-test, correlation coefficient, SEM) were performed in Excel. All graphs were assembled using the free and open-source software GNU Image Manipulation Program (GIMP, <https://www.gimp.org/>) and Inkscape (<https://inkscape.org/>). Proposed model for collaboration (Fig. 6) was assembled using BioRender (<https://app.biorender.com/>).

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Author Contributions:

M.D.R. and R.A.C. designed the experiments and wrote the manuscript. M.D.R. performed the experiments, and M.D.R. and R.A.C. analyzed the data.

Conflict of Interest

The authors declare no conflict of interest.

Data Availability

Datasets regarding the raw data and analysis of invasion assays, fluorescence microscopy and associated kinetic analyses are available in Dryad (<https://doi.org/10.5061/dryad.0k6djhb0g>).

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975

Figure 1: Deletion of TarP and TmeA impair invasion and attenuates kinetics of actin recruitment and turnover.

(A,B) Cos7 cells were transfected with Actin-GFP or mRuby-LifeAct for 24 hours prior to infection with wild-type, single-knockout (Δ TarP, Δ TmeA), cis-complemented (*cis*-TarP, *cis*-TmeA), or double-knockout (Δ TmeA/ Δ TarP) *Chlamydia* strains at MOI=20. Infection was monitored by live-cell confocal microscopy using a Nikon CSU-W1 spinning disk microscope, obtaining images every 20 seconds for 30 minutes to identify sites exhibiting actin recruitment. (B) Recruitment events were isolated by selecting regions containing invading *Chlamydia* and elevated actin fluorescence. Background fluorescence was subtracted, and fold recruitment was calculated as a function of the fold increase in mean fluorescence intensity of recruited actin compared to basal actin fluorescence. Detailed visualization of this process can be found in Fig. S1. Data is displayed as mean fold recruitment for each timepoint $\pm$ SEM compiled from a minimum N=18 recruitment events. (C) HeLa cells were infected with each *Chlamydia* strain indicated above at MOI=50 and stained using the “in-and-out” method which distinguishes non-internalized EBs from total cell-associated EBs, as described in Materials and Methods. Results were normalized against mean invasion efficiency of wild-type *Chlamydia* and plotted as normalized mean $\pm$ SEM. Data was collected from 15 fields, with each field containing an average of 100 *Chlamydiae*. Statistical significance was determined by T- test. (D,E) All actin recruitment events used to create the averaged plot shown previously (Fig. 1B) were individually divided into recruitment and turnover phases (Fig. S2). Individual rates of (D) recruitment and (E) turnover were plotted on a violin plot with inset boxplot, reporting the median rate $\pm$ SD for each strain tested. Violin plots contain a minimum N=18 individual rates. Statistical significance was determined by Wilcoxon Rank-sum. All data are representative of at least 3 independent experiments, * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$.

Figure 2: Deletion of TarP and TmeA exerts divergent effects on Arp2/3 kinetics.

(A,B) Cos7 cells were transfected with Arp3-GFP or Arp3-mCherry for 24 hours prior to infection with wild-type, single-knockout (Δ TarP, Δ TmeA), cis-complemented (*cis*-TarP, *cis*-TmeA), or double-knockout (Δ TmeA/ Δ TarP) *Chlamydia* strains at MOI=20. Infection was monitored by live-cell confocal microscopy using a Nikon CSU-W1 spinning disk microscope, obtaining images every 20 seconds for 30 minutes to identify sites exhibiting Arp3 recruitment. (B) Fluorescence intensity of Arp3 recruitment was measured as described earlier (Fig. 1B) and plotted as mean fold recruitment for each timepoint +/- SEM compiled from a minimum N=12 recruitment events. (C,D) Kinetics of Arp3 recruitment and turnover were analyzed for each strain indicated above using the same methodology described in Fig. 1D,E. Violin plots contain a minimum N=12 individual rates, reporting the median rate +/- SD. Statistical significance was determined by Wilcoxon Rank-sum. All data are representative of at least 3 independent experiments, * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$.

Figure 3: Deletion of TarP prevents recruitment of Fmn1 during invasion

(A,B) Cos7 cells were transfected with formin 1 isoform 1B fused to GFP (Fmn1-GFP) or mCherry (Fmn1-mCherry) for 24 hours prior to infection with wild-type, single-knockout (Δ TarP, Δ TmeA), cis-complemented (*cis*-TarP, *cis*-TmeA), or double-knockout (Δ TmeA/ Δ TarP) *Chlamydia* strains at MOI=20. Infection was monitored by live-cell confocal microscopy using a Nikon CSU-W1 spinning disk microscope, obtaining images every 20 seconds for 30 minutes to identify sites exhibiting Fmn1 recruitment. (B) Fluorescence intensity of Fmn1 recruitment was measured as described earlier (Fig. 1B) and plotted as mean fold recruitment for each timepoint +/- SEM compiled from a minimum N=15 recruitment events. (C,D) Kinetics of Fmn1 recruitment and turnover were analyzed for each strain indicated above using the same methodology described in Fig. 1D,E. Violin plots contain a minimum N=15 individual rates, reporting the median rate +/- SD. Statistical significance was determined by Wilcoxon Rank-sum. All data are representative of at least 3 independent experiments, * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$.

Figure 4. Formin and Arp2/3 activity are necessary for efficient invasion and optimal actin recruitment kinetics.

(A) Cos7 cells were transfected with GFP-Fmn1, mEmerald-mDia1, or mEmerald-mDia2 for 24hrs prior to infection with *Chlamydia* stained with CMTPIX at MOI=20. Infection was monitored by live-cell confocal microscopy using a Leica SD6000 AF Spinning Disc microscope with a 60x (NA 1.40) objective. Images were obtained every 20 seconds for 30 minutes and compiled into videos in ImageJ to identify sites exhibiting colocalization of host formins (GFP) and *Chlamydia* (RFP). (B) HeLa cells were mock-treated or pretreated with inhibitors against formin (10 μ M SMIFH2), Arp2/3 (100 μ M CK666) or both (CK+SMI) for 1hr, infected with *C. trachomatis* serovar L2 at MOI=50 and stained using the “in-and-out” method which distinguishes non-internalized EBs from total cell-associated EBs, as described in Materials and Methods. Results were normalized against mean invasion efficiency of mock treated cells (Untreated mean = 49% invasion efficiency) and plotted as normalized mean $\pm$ SEM. Data was collected from 20 fields, with each field containing an average of 108 Chlamydiae. Statistical significance was determined by T- test. (C) Cos7 cells were transfected with GFP-actin or a GFP empty vector plasmid for 24hrs prior to mock-treatment or pretreatment with 10 μ M SMIFH2, 100 μ M CK666, or both for 1hr. Transfected cells were infected with *Chlamydia* stained with CMTPIX at MOI=20 and imaged by quantitative live-cell imaging, collecting images every 20 seconds for 30 minutes. Fluorescence intensity of GFP-actin recruitment was measured as described earlier (Fig. 1B) and plotted as mean fold recruitment for each timepoint $\pm$ SEM compiled from a minimum N=16 recruitment events. (D,E) Kinetics of GFP-actin recruitment and turnover were analyzed for each condition using the same methodology described in Fig. 1D,E. Violin plots contain a minimum N=16 individual rates, reporting the median rate $\pm$ SD. Statistical significance was determined by Wilcoxon Rank-sum. All data are representative of at least 3 independent experiments, * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$.

Figure 5: Formin 1 and Arp3 collaboration enhances recruitment kinetics at entry sites.

(A,B) Cos7 cells were transfected with (A) GFP-formin 1 isoform 1B (GFP-Fmn1) or (B) GFP-Arp3 for 24 hours prior to mock-treatment or pretreatment with 10 μ M SMIFH2 or 100 μ M CK666 for 1 hour. Quantitative live-cell imaging of invading CMTPX- labeled *Chlamydia* (MOI=20) was performed as described in Fig. 1B. Briefly, images were acquired once every 20 seconds for 30 minutes and assembled into videos. MFI of recruitment events were obtained, and background fluorescence subtracted, before quantifying and plotting the fold recruitment values $\pm$ SEM of Fmn1 or Arp3 for each timepoint. Data was compiled from a minimum N=16 recruitment events. (C) Cos7 cells were co-transfected with GFP-Fmn1 and mCherry-Arp3 for 24 hours prior to infection with unlabeled *C. trachomatis* (MOI=20) followed by quantitative live-cell imaging of invasion. Image acquisition and processing was performed as described above with a few modifications. Since CMTPX and mCherry-Arp3 fluorescence overlap, unlabeled *Chlamydia* was used, thus recruitment events are defined simply as regions containing elevated GFP-Fmn1 fluorescence compared to local background. MFI of both GFP-Fmn1 and mCherry-Arp3 were obtained from the recruitment ROI, and background was subtracted from both GFP and RFP channels independently. Maximal MFI was derived from both channels and used to normalize fluorescence as percent maximal MFI for each protein. Normalized MFI values for each timepoint were compiled from 33 events, plotting mean %Max MFI $\pm$ SEM. (D) Recruitment of mEmerald-mDia1 and mEmerald-mDia2 was monitored in the presence and absence of 100 μ M CK666 using the method described above for monitoring the recruitment of GFP-Fmn1 and GFP-Arp3. Data is displayed as mean fold recruitment for each timepoint $\pm$ SEM compiled from a minimum N=22 recruitment events. (E,F) Kinetics of Fmn1 and Arp3 recruitment and turnover were analyzed in the presence and absence of 100 μ M CK666 or 10 μ M SMIFH2, respectively, using the same methodology described in Fig. 1D,E. Violin plots contain a minimum N=16 individual rates, reporting the median rate $\pm$ SD. Statistical significance was determined by

Wilcoxon Rank-sum. All data are representative of at least 3 independent experiments, * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$.

Figure 6: Proposed model for collaboration between formin and Arp2/3 and subsequent rapid actin recruitment and turnover.

C. trachomatis engages and activates a multitude of host receptors whose activation is linked to the recruitment of formin, Arp2/3, and actin in non-invasion contexts. It is currently unknown whether receptor activation contributes to actin remodeling during invasion. Concurrent with receptor engagement, *Chlamydia* secretes the effectors TmeA and TarP via Type III secretion system; each effector activates Arp2/3 through N-WASP or WAVE2, respectively. Deletion of either TarP or TmeA attenuated the kinetics of Arp2/3 recruitment and turnover, which may reflect a failure to optimally activate and integrate Arp2/3 into actin networks. In addition to recruiting Arp2/3, TarP was also shown to be essential for the recruitment of Fmn1 and may also function to recruit mDia1 and/or mDia2 via signaling through Rac1. Collaboration between Arp2/3 and formin via their respective actin branching and elongating activities increases the rate of actin recruitment. Inhibition of Arp2/3 by CK666 or formin by SMIFH2 prevents this collaboration and constrains the actin network into either filamentous-only or branching-only networks. The actin network generated by *Chlamydia* is rapidly turned over alongside the actin nucleators found within it. Although the precise mechanism by which this process occurs remains unknown, several actin depolymerization factors, including ADF/Cofilin, have been implicated by an siRNA screen to participate in invasion.

Figure S1: Detailed image analysis throughput used to evaluate the intensity of protein recruitment.

Quantitative live-cell imaging of fluorescent protein recruitment was utilized several times in various conditions throughout this study, necessitating a consistent throughput to facilitate direct comparison. (A) Videos of *Chlamydia* invasion were assembled following image acquisition in ImageJ and analyzed to identify protein recruitment events. Protein recruitment was defined by regions exhibiting high fluorescence intensity (Yellow) as compared to local background (Red). When possible, fluorescent *Chlamydia* (CMTPIX-*Chlamydia*, GFP-*Chlamydia*, RFP-*Chlamydia*) were used to ensure recruitment was specific to an invading bacterium. Regions-of-interest (ROIs) specific to recruitment were selected to include the entirety of the recruitment event across all timepoints, and a concentric region immediately outside of this region was selected as a background ROI. (B) The mean fluorescence intensity (MFI) of both recruitment and background ROI was obtained for each timepoint. (C) Background MFI was subtracted from recruitment MFI to normalize for local variations in fluorescence intensity and for photobleaching during image acquisition. Fold recruitment was calculated by dividing the MFI of each timepoint by the average MFI of 5 timepoints (100 sec, yellow dots) prior to the start of recruitment, defined as the point in which recruitment velocity begins to increase. (D) Fold recruitment values were plotted against time for each recruitment event. Multiple events were aligned according to peak recruitment for each event before calculating the mean fold recruitment across all events.

Figure S2: Entry and exit points for each phase determined by velocity of recruitment and turnover.

Each event used to generate the protein recruitment plots shown throughout this study were divided into three phases, recruitment, fast turnover, and slow turnover, in order to study their kinetics. Entry and exit points for these phases was determined by plotting the first derivative value (i.e., velocity) of protein recruitment for each timepoint. Start of recruitment was defined as 20 seconds after velocity began to increase (t=160s, dark green), since this timepoint was more consistently associated with recruitment

across events than the preceding timepoint (t=140s). End of recruitment was defined as the timepoint immediately prior to when velocity is at its minimum, or 20 seconds prior to Vmin (t=240s, light green). Start of fast turnover was defined as the timepoint associated with Vmin (t=260s, dark gray), and the end of fast turnover was defined as the point in which turnover ceased to decelerate (t=340s, light gray). Start of slow turnover was defined as the timepoint immediately following the end of fast turnover (t=360s, dark orange), and terminated at the timepoint prior to when velocity crosses the x-intercept (t=760s, light orange). This analysis was conducted for all events independently and was used as the basis for linear regression analysis to determine the rate (slope) of recruitment, fast turnover, and slow turnover across multiple proteins, strains, and inhibitor treatment conditions.

Figure S3: Linear regression analysis of population-level kinetics derived from effector deletion strains.

All protein recruitment events used to generate the averaged plots shown throughout the study (Fig. 1B, Fig. 2B, Fig. 3B) were individually divided into recruitment and turnover phases (Fig. S2). Fold recruitment values were averaged for each timepoint and analyzed by linear regression to ascertain the population-level kinetics of recruitment and turnover for each condition. Averaged rate slopes were divided according to the dataset they correspond to, summarizing the rate distributions depicted as violin plots throughout the study into comprehensive linear slopes which represent the rate of protein recruitment and turnover. Statistical significance was determined by t-test, * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$.

Figure S4: Actin turnover duration is reduced following deletion of TarP and TmeA.

Actin turnover velocity of (A) wild-type, (B,C) cis-complemented (*cis*-TarP, *cis*-TmeA), (D,E) single-knockout (Δ TarP, Δ TmeA), or (F) double-knockout (Δ TmeA/ Δ TarP) *Chlamydia* strains were plotted for each timepoint. T=0 represents maximal fold recruitment of actin for each strain, with the termination of actin turnover defined by the timepoint immediately before actin velocity crosses the X-intercept (yellow dot).

(G) Fold recruitment values were plotted against time for each strain, highlighting the timepoint denoting the end of actin turnover for each strain. Protein turnover duration was quantified for each recruitment event monitored across all strains and proteins utilized throughout the study.

Figure S5: SMIFH2 does not inhibit myosin superfamily proteins at relatively low concentrations.

Cos7 cells were treated with (A) the nonmuscle myosin II inhibitor s-nitro blebbistatin, (B) the rho-associated protein kinase (ROCK) inhibitor Y27632, or (C) the pan-formin inhibitor SMIFH2 at various concentrations for 1 hour. Cells were probed with anti-vinculin and anti-paxillin antibodies and stained using fluorescent secondary antibodies to identify the integrity of focal adhesions following inhibitor treatment. (D) Cos7 cells were treated with blebbistatin, Y27632, or SMIFH2 at various concentrations for 1 hour prior to lysis in Laemmli buffer. Lysates were resolved by SDS-PAGE followed by Western blotting and probed with antibodies reactive against phospho-myosin light chain 2 (p-MLC2 Thr18/Ser19), total myosin light chain 2 (t-MLC2), and GAPDH. (E) p-MLC2 abundance was quantified for each sample by ratiometric analysis of p-MLC2 intensity versus total MLC2 intensity, with each condition normalized against mock treatment. The concentration of SMIFH2 utilized to inhibit formins throughout this study (10 μ M) did not prevent the formation of intact focal adhesions or restrict phosphorylation of MLC2.

Figure S6: Linear regression analysis of population-level kinetics following inhibition of formin or Arp2/3.

All protein recruitment events used to generate the averaged plots shown throughout the study (Fig. 4C, Fig. 5A,B,D) were individually divided into recruitment and turnover phases (Fig. S2). Fold recruitment values were averaged for each timepoint and analyzed by linear regression to ascertain the population-level kinetics of recruitment and turnover for each condition. Averaged rate slopes were divided according to the dataset they correspond to, summarizing the rate distributions depicted as violin plots throughout

the study into comprehensive linear slopes which represent the rate of protein recruitment and turnover.

Statistical significance was determined by t-test, * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$.

Figure S7: Kinetics of mDia1 and mDia2 recruitment and turnover are attenuated by Arp2/3 inhibition.

All mDia1/2 recruitment events used to create the averaged plot shown previously (Fig. 2D) were individually divided into recruitment, fast turnover and slow turnover phases (Fig. S2). (D) Individual rates of recruitment were plotted on a violin plot with inset boxplot, reporting the median rate $\pm$ SD for each inhibitor treatment condition. (E) Individual rates of fast turnover (Left) and slow turnover (Right) were plotted using the same method as recruitment, with fast and slow turnover plots grouped together according to condition. Violin plots contain a minimum N=22 individual rates. Statistical significance was determined by Wilcoxon Rank-sum. Data are representative of at least 3 independent experiments, ** $P \leq 0.01$, *** $P \leq 0.001$.

Table S1: Statistical values associated with recruitment and turnover of actin following effector deletion

Table S2: Statistical values associated with recruitment and turnover of Arp3 following effector deletion

Table S3: Statistical values associated with recruitment and turnover of formin 1 following effector deletion

Table S4: Statistical values associated with recruitment and turnover of actin following inhibitor treatment

Table S5: Statistical values associated with recruitment and turnover of formin 1 and Arp3 following inhibitor treatment

Table S6: Statistical values associated with recruitment and turnover of mDia1/2 in presence and absence of CK666

Table S7: Pairwise correlation analyses of mDia1/2 kinetics in presence and absence of CK666

Video S1: Representative video of fluorescent protein recruitment during *Chlamydia* invasion

Cos7 cells were transfected with plasmids expressing various GFP-fusion proteins for 24 hours prior to infection with CMTPX-stained *C. trachomatis* EBs (MOI=20). Bacterial adhesion and entry was monitored by live-cell imaging using a spinning disk confocal microscope (Nikon CSU-W1 or Leica SD6000 AF), obtaining images every 20 seconds for 30 minutes to identify sites exhibiting protein recruitment. Images were assembled into videos in ImageJ and annotated to highlight recruitment events. The representative video shown here depicts GFP-actin recruitment at sites where CMTPX-*Chlamydia* contacts the host cell (arrows). Similar videos were assembled for all proteins monitored throughout the study, each yielding recruitment events that exhibit tight localization around fluorescent *Chlamydia*, enabling straightforward quantification of recruitment intensity and duration (Figs. S1-3).

Video S2: Co-inhibition of formin and Arp2/3 yields abnormal actin recruitment kinetics around invading *Chlamydia*

Cos7 cells were transfected with GFP-actin 24hrs prior to pretreatment with 10 μ M SMIFH2 and 100 μ M CK666 for 1hr. Transfected cells were infected with unlabeled *Chlamydia* at MOI=20 and imaged by quantitative live-cell imaging, collecting images every 20 seconds for 30 minutes. Images were assembled into videos in ImageJ and annotated to highlight recruitment events (arrows).

Figure 1

Figure 1

Figure 2

Figure 2

Figure 3

Figure 3

Figure 4

Figure 4

Figure 5

Figure 5

Figure 6

Figure 6