

# **ACD-crosslinked actin oligomers are potent toxins inhibiting formin-mediated actin polymerization**

David Heisler – Hayes Forum

This work has been previously published (Heisler et al., 2015). Additional figures, experimental methods, supplementary data/figures, and supplemental videos can be found at:

<http://science.sciencemag.org/content/349/6247/535.long>

## **Abstract:**

Bacterial toxins are the deadliest compounds on the planet; a single copy of a toxin is capable of compromising a host cell. Amplification of toxicity is typically achieved by enzymatically targeting signaling cascades or inhibiting vital host proteins/complexes present in relatively few copies. Due to its fundamental importance for a living cell, a major cytoskeletal protein, actin, is a common target of many toxins. However, due to the high abundance of actin in the cytosol it is not clear how actin-targeting toxins can achieve their high efficiency. One of such toxins produced by pathogenic strains of *Vibrio cholerae*, *Vibrio vulnificus*, and *Aeromonas hydrophila*, the actin crosslinking domain (ACD), catalyzes the formation of an amide bond between Lys-50 and Glu-270 of actin monomers, resulting in the formation of actin oligomers, which are unable to function properly. It was believed that ACD toxicity stems from a slow failure of the cytoskeleton due to the gradual accumulation of non-functional actin oligomers. However, this mechanism can be effective only assuming intracellular delivery of high concentrations of ACD toxin, because the *in vitro* rate of ACD activity applied to cellular conditions suggests that a single ACD molecule would require over 6 months to crosslink half of all cytoplasmic actin. On the contrary, we found that to implement its cellular toxicity, ACD is not required to crosslink all actin in the cell: the integrity of intestinal cell monolayers is compromised dramatically when only a small fraction (<6%) of the total cellular actin is crosslinked. This suggests that ACD-crosslinked actin oligomers even at low doses are toxic to cells.

Since actin-binding domains of some actin-regulatory proteins are organized in tandems, these proteins potentially can bind to actin oligomers with affinities higher than those for a G-actin monomer, due to multiple binding sites accessible on a single oligomer molecule. This would render these actin-regulatory proteins non-functional. Formins are one family of such proteins governing the actin cytoskeleton dynamics important for numerous cellular processes. The main functional domains of formins, formin homology domains 1 (FH1) and 2 (FH2), cooperate in nucleation and elongation of actin filaments. Our data indicate that formins preferentially bound to the crosslinked actin oligomers with abnormally high affinity in cell culture. We found that actin polymerization controlled by formins was inhibited by sub-nanomolar concentrations of actin oligomers. To investigate the mechanism of formin inhibition exerted by the ACD-crosslinked actin oligomers, we performed total internal fluorescence reflection microscopy (TIRFM) to monitor actin polymerization at the individual filament level. In the presence of profilin, the oligomers caused reversible blocks of elongation of formin-controlled, but not formin-free, filaments. The persistence of the blocks, as well as the fraction of blocked filaments was dependent upon oligomer concentration as well as the length of the FH1 domains, suggesting that both FH1 and FH2 domains of formin contribute to the inhibition in profilin-dependent and profilin-independent manners, respectively. Mathematical modeling of polymerization in bulk (pyrene actin assays) and on a single filament level (TIRFM) revealed that the oligomers potently inhibit both nucleation and elongation steps of actin filament assembly controlled by formins. Therefore, our findings implicate that ACD employs a novel toxicity mechanism by converting cytoplasmic actin into highly toxic oligomers that specifically target key steps of actin dynamics. This implies that toxins can not only exploit existing signaling pathways but also initiate a new toxicity cascade with de novo produced crosslinked actin species as “second messengers.” ACD creates toxic

derivatives of actin with a disruptive “gain-of-function” mode of operation. These new actin species bind with high affinity to formins and adversely affect both their nucleation and elongation. We propose that the seemingly straightforward original assumption that ACD acts by the accumulation of bulk amounts of nonfunctional actin is inaccurate or at least incomplete. The toxin can be highly efficient at very low concentrations by acting on formins and, potentially, other actin regulatory proteins. This finding calls for the careful reevaluation of mechanisms used by other actin related toxins, both of protein and small-molecule natures.

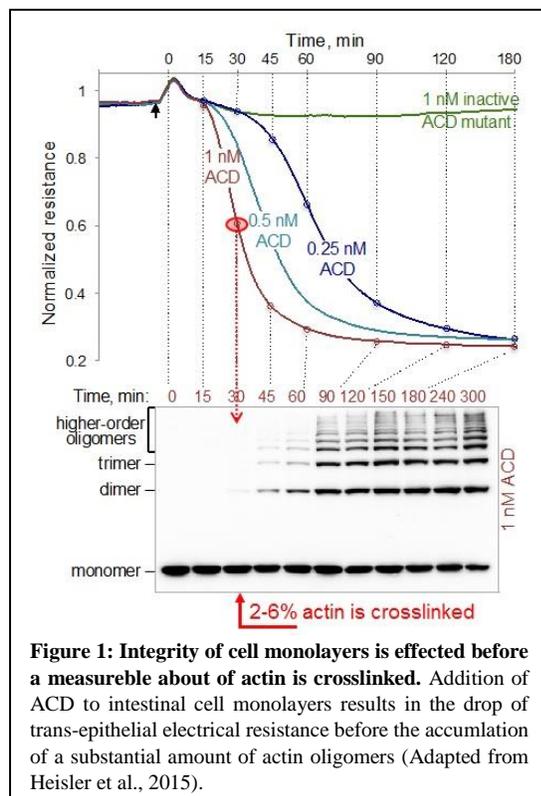
Bacterial toxins are the deadliest compounds on Earth; a single copy of some toxins are sufficient to compromise or even kill a host (Tam & Lingwood, 2007). Typically, bacterial toxins amplify their toxicity by regulating the activity of signaling cascades (e.g. over activation of Rho-GTPase signaling cascades by cholera and anthrax toxins (Young & Collier, 2007)) or by inactivation of low abundant, essential host complexes (e.g. inactivation of ribosomes by Shiga and diphtheria toxins (M. S. Lee, Koo, Jeong, & Tesh, 2016)). Bacteria must produce efficient toxins because i) the host immune system rapidly neutralizes bacteria and their toxins; ii) only a small amount of toxin-producing bacterial cells are present early upon infections; iii) host commensal bacteria prevent colonization.

Due to its vital role in various cellular processes, actin is a common target of bacterial toxins (Barbieri, Riese, & Aktories, 2002). Actin-targeting toxins work through direct and indirect modifications of actin dynamics. Actin is found in a delicate equilibrium between monomeric (G-actin) and filamentous (F-actin) states, which allows for the constant rearrangement of the host’s cytoskeleton. This equilibrium and rearrangement is regulated by a wide variety of actin regulatory proteins. However, direct modification of actin by bacterial toxins (i.e. ADP-ribosylation (Barth

& Aktories, 2011), binding of SipA (Lilic et al., 2003), or nucleation of new filaments by VopL (Namgoong et al., 2011)), can shift this equilibrium in either direction, disrupting the host's cytoskeleton. Additionally, bacterial toxins effect this equilibrium by altering the normal state of signaling cascades that also control actin dynamics, such as covalent modifications of Rho-GTPases (Lerm, Schmidt, & Aktories, 2000) and proteins the control the equilibrium (W. L. Lee, Grimes, & Robinson, 2015).

Upon delivery to the cytoplasm of effected host cells, the actin crosslinking domain (ACD) is an actin-specific toxin that catalyzes the covalent cross-linking of Lysine-50 (K50) of one actin monomer, to Glutamate-270 (E-270) of another actin monomer through the formation of an amide peptide bond (Cordero, Kudryashov, Reisler, & Satchell, 2006; Fullner & Mekalanos, 2000). This cross-link leads to the formation of actin oligomers of various sizes (Cordero et al., 2006; Kudryashov, Cordero, Reisler, & Satchell, 2008; Kudryashov, Durer, et al., 2008). Typically, K50

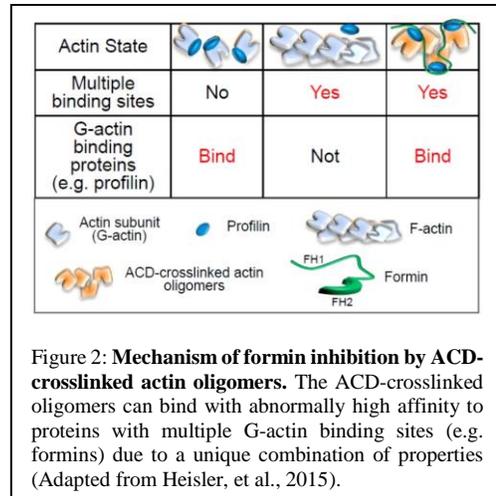
and E270 are located approximately 20 Å apart in F-actin; however, the ACD-mediated cross-linking results in a cross-link that disrupts this F-actin inter-subunit interface, making the actin oligomers non-polymerizable (Kudryashov, Durer, et al., 2008). This lead to the previously accepted hypothesis that ACD toxicity is dependent upon the accumulation of bulk amounts of actin into non-functional oligomers and the compromising of the host cell's cytoskeleton.



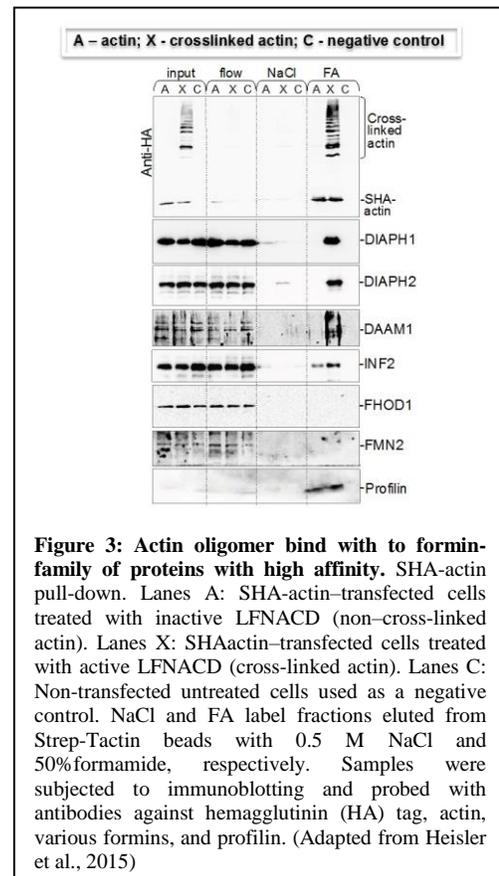
However, actin is one of the most abundant proteins in a eukaryotic cell, with concentrations

exceeding 100  $\mu\text{M}$  (Pollard & Borisy, 2003). Using previously determined rates of *in vitro* ACD cross-linking rates (Kudryashova, Kalda, & Kudryashov, 2012), it would take over six months for a single copy of the ACD toxin to cross-link half the actin cytoskeleton. Typically, ACD is found in pathogenic, Gram-negative bacteria from the *Vibrio* and *Aeromonas* spp., which result in a compromised host rapidly after colonization of a bacterium. In agreement with this quickly compromised host cells, the integrity of intestinal cell monolayers (IEC-18 cell lines) was drastically effected when only a small fraction ( $\sim 5\%$ ) of cellular actin was cross-linked (Figure 1; (Heisler et al., 2015)).

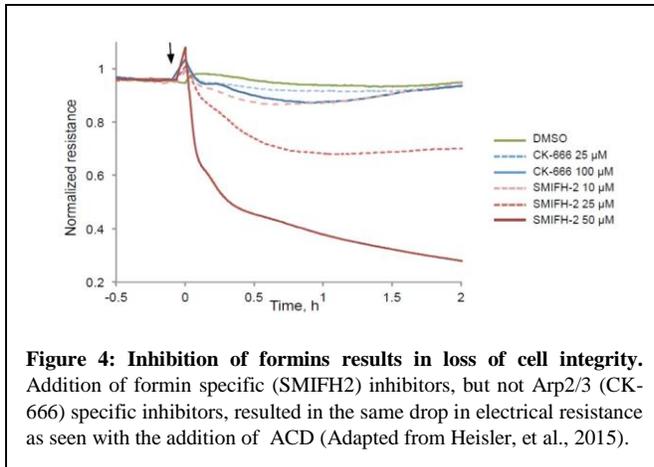
We hypothesized that this drastic drop in resistance was due to the actin oligomers binding with high affinity to actin binding proteins and inhibiting their activity. In support of this hypothesis, the actin oligomers possess a unique combination of properties that are not found in F- or G-actin, but rather they contain properties of each state of actin. The oligomers are capable of still binding G-actin binding proteins, like profilin (Cordero et al., 2006), but contain multiple binding sites, similar to F-actin (Figure 2; (Heisler et al., 2015)). This combination of properties would allow for multivalent binding of the oligomers to actin regulatory proteins with multiple actin



**Figure 2: Mechanism of formin inhibition by ACD-crosslinked actin oligomers.** The ACD-crosslinked oligomers can bind with abnormally high affinity to proteins with multiple G-actin binding sites (e.g. formins) due to a unique combination of properties (Adapted from Heisler, et al., 2015).



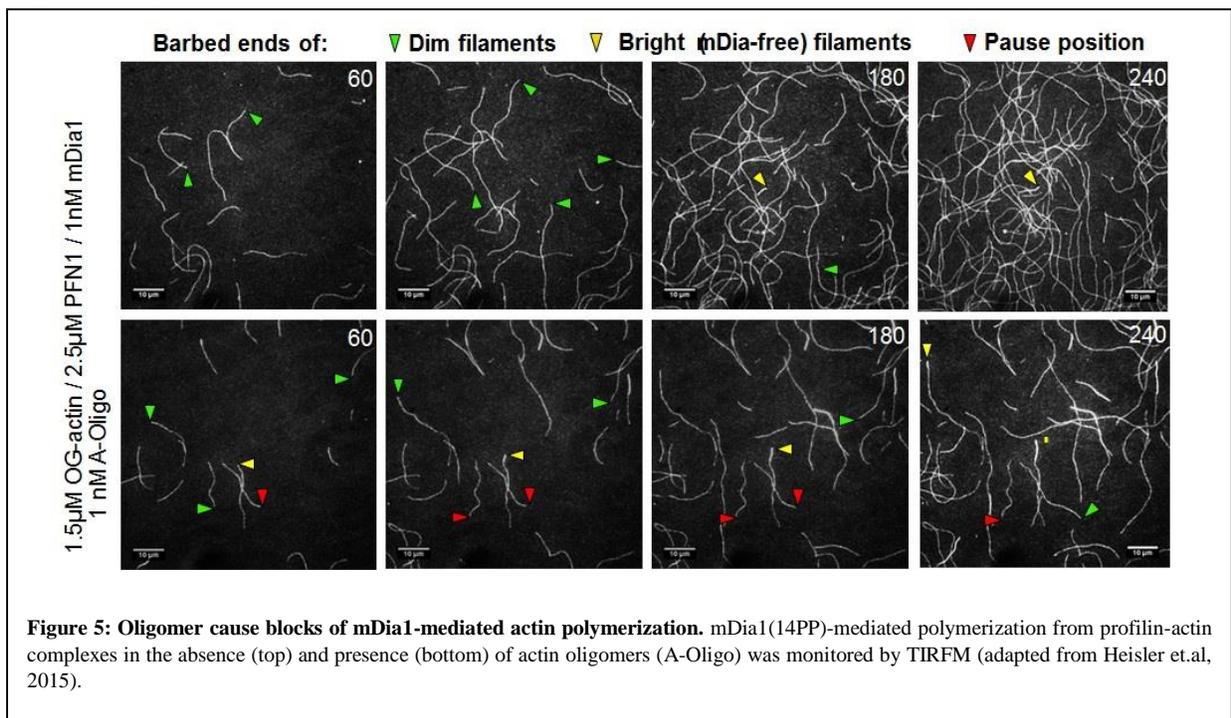
**Figure 3: Actin oligomer bind with to formin-family of proteins with high affinity.** SHA-actin pull-down. Lanes A: SHA-actin-transfected cells treated with inactive LFNACD (non-cross-linked actin). Lanes X: SHA-actin-transfected cells treated with active LFNACD (cross-linked actin). Lanes C: Non-transfected untreated cells used as a negative control. NaCl and FA label fractions eluted from Strep-Tactin beads with 0.5 M NaCl and 50% formamide, respectively. Samples were subjected to immunoblotting and probed with antibodies against hemagglutinin (HA) tag, actin, various formins, and profilin. (Adapted from Heisler et al., 2015)



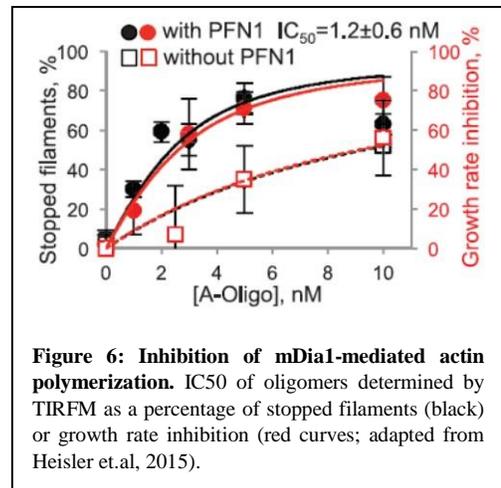
binding domains. The affinity (avidity) of the oligomers would be the results of simultaneous binding of multiple actin binding domains, making the affinity a multiple of each actin binding domain.

We utilized the anthrax toxin delivery machinery (Milne, Blanke, Hanna, &

Collier, 1995) to deliver ACD (LF<sub>N</sub>ACD; (Cordero et al., 2006)) into HeLa cells which had been transfected with a double-tagged, Twin-Strep-tag II and hemagglutinin, actin (SHA-actin; a gift from T. Vitta and M. Vartiainen, University of Helsinki) and used these cells for a pull-down assay against cross-linked actin oligomers. Several proteins from the formin family of proteins, including DIAPH1, DIAPH2, DAAM1, and INF2, preferentially bound to the ACD-cross-linked actin oligomers (Figure 3). Formins are a major family of actin binding proteins involved in cell migration, cytokinesis, cell-to-cell contacts, mitochondrial fission, stress fiber stabilization, others

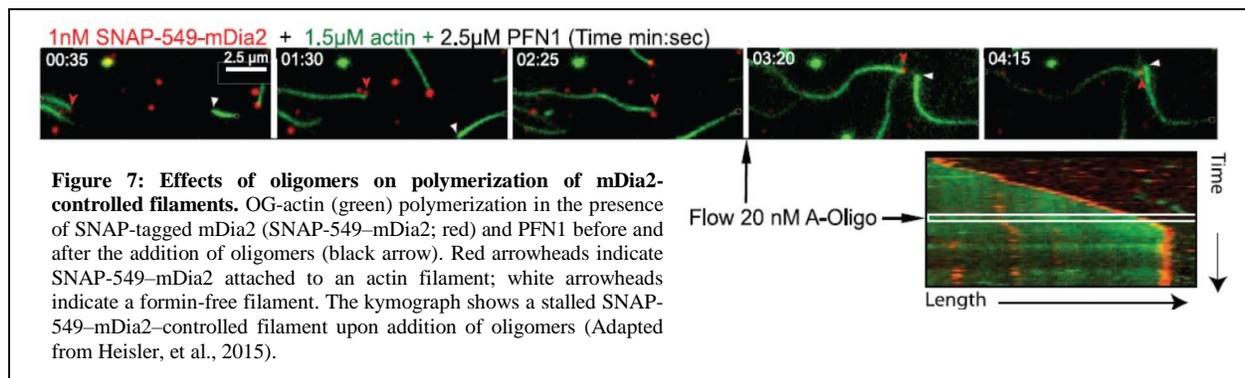


other vital cellular functions (Breitsprecher & Goode, 2013). Formins are characterized by their two functional domains, formin homology domains 1 (FH1) and 2 (FH2), which cooperate in the nucleation and elongation of actin filaments (Kovar, 2006). The noncovalent FH2/FH2 homodimer nucleates new filaments and remains processively attached to the barbed end, fast



growing end of the filament while also protecting the filament from capping proteins (Moseley, Maiti, & Goode, 2006). Tandem poly-proline (PP) stretches within the FH1 domains bind actin-profilin complexes and can accelerate elongation up to 10-fold (Kovar, Harris, Mahaffy, Higgs, & Pollard, 2006). To determine if the inhibition of formins could explain the loss the cell integrity, we used SMIFH2, a formin specific inhibitor (Rizvi et al., 2009), on cell monolayers. Similar to ACD, the small molecule inhibitor resulted in a similar drop in cell integrity, shortly after addition (Figure 4).

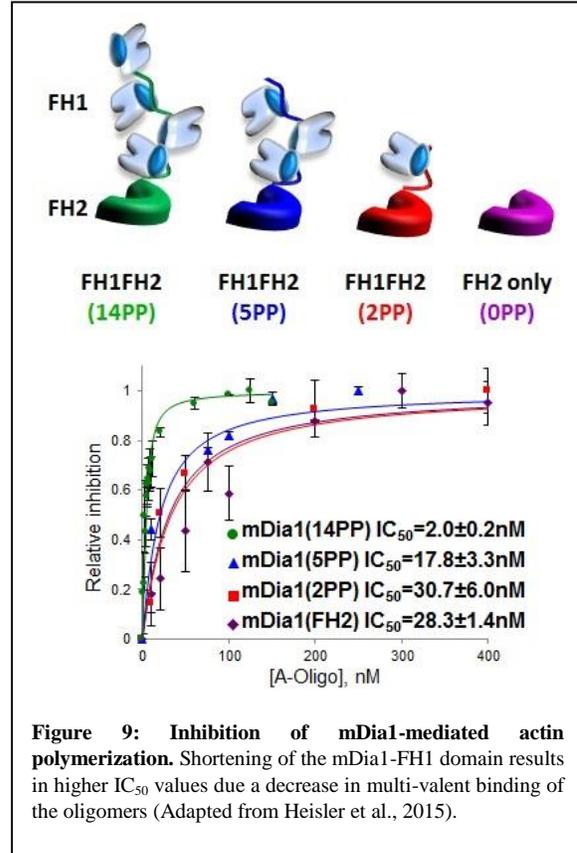
To elucidate the mechanism of formin inhibition, we utilized constitutively active fragments of the mouse orthologs mDia1 and mDia2 (FH1-FH2 domain fragments). Using these constructs, we monitored actin polymerization at the single filament level by total internal reflection fluorescence (TIRF) microscopy. To visualize individual actin filaments and the formins attached



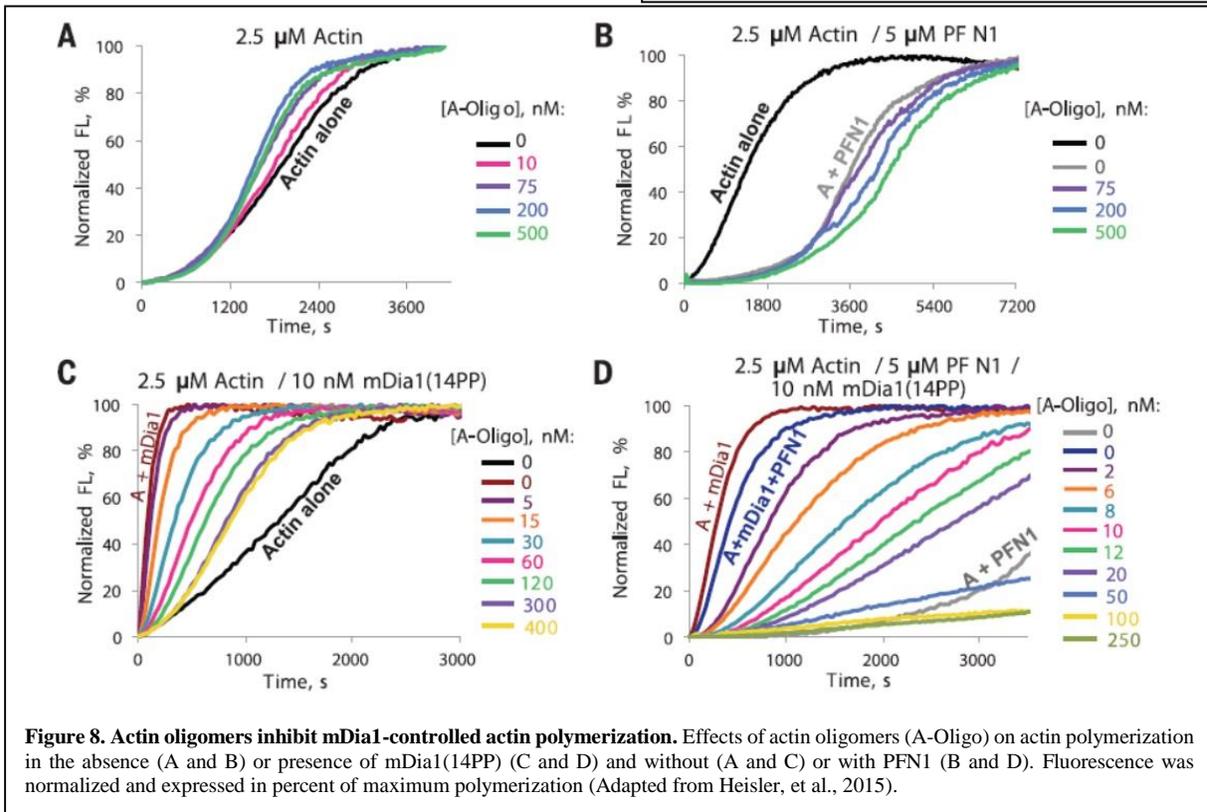
to filaments, 33% of total actin monomers were labeled with Oregon-green (OG-actin). Individual molecules of mDia2 were visualized through the fusion of a SNAP-tag® domain (New England Biolabs) and subsequent labeling with SNAP-Surface 549. The addition of actin oligomers caused reversible blocks of mDia1- and mDia2-mediated actin polymerization in the presence of profilin (Figure 5 and 7). Formin-controlled filaments were identified by faster growth and their dimmer appearance than formin-free actin filaments (Kovar et al., 2006). The fraction of blocked mDia1-mediated polymerizing filaments and the average growth rates depended on the concentration of actin oligomers and resulted in a median inhibitory concentration ( $IC_{50}$ ) of  $1.2 \pm 0.6$  nM (Figure 6). Also, we observed numerous stopped filaments that would begin to polymerize with rates characteristic of formin-controlled filament due to the likely dissociation of a formin-bound actin oligomer (Figure 5). We also confirmed the inhibition of mDia2, a formin with only two polyproline rich stretches in its FH1 domains. Likewise, the actin oligomers potently inhibited elongation of mDia2-controlled actin filaments (Figure 7), proving the oligomer's inhibition of formins is not specific to mDia1. The inhibition was not as significant in the absence of profilin,

but still resulted in measurable blocks of formin-controlled actin polymerization (Figure 6). The interaction of the oligomer with profilin and the polyproline stretches of FH1 domain is therefore not an absolute requirement; rather the oligomer-profilin-polyproline interaction significantly amplifies the efficiency by contributing to the multisite binding of the oligomers. In agreement, the  $appK_I$  for mDia2 (containing 2 polyproline stretches) was higher than that found for full length mDia1(14PP) (data can be found in figure 3 of Heisler et al, 2015).

To further characterize the mechanism of inhibition, we utilized bulk pyrene actin



**Figure 9: Inhibition of mDia1-mediated actin polymerization.** Shortening of the mDia1-FH1 domain results in higher  $IC_{50}$  values due a decrease in multi-valent binding of the oligomers (Adapted from Heisler et al., 2015).

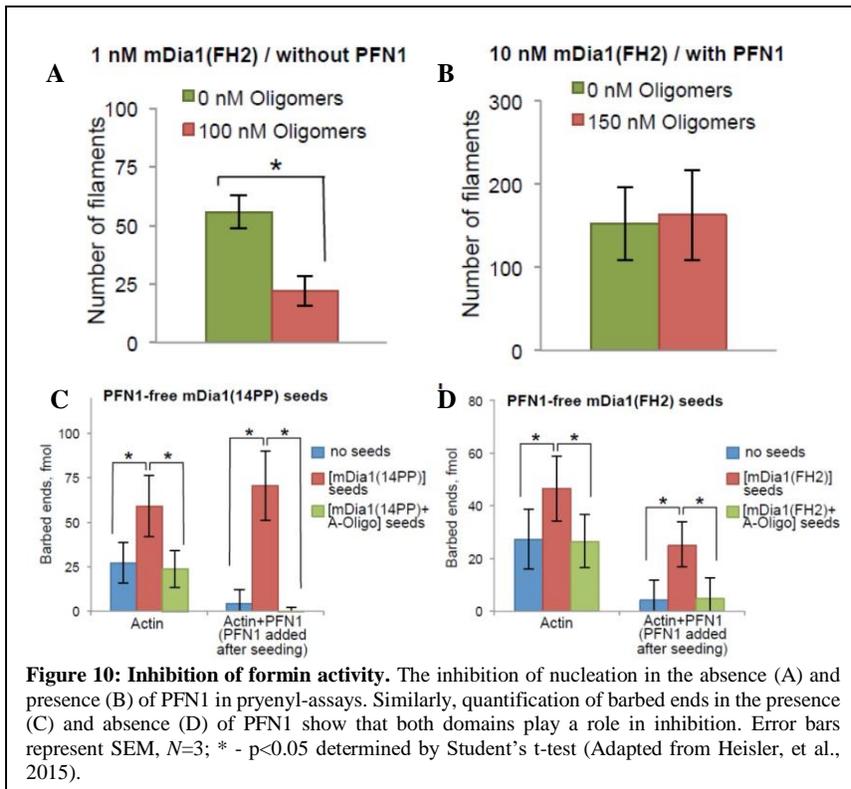


**Figure 8. Actin oligomers inhibit mDia1-controlled actin polymerization.** Effects of actin oligomers (A-Oligo) on actin polymerization in the absence (A and B) or presence of mDia1(14PP) (C and D) and without (A and C) or with PFN1 (B and D). Fluorescence was normalized and expressed in percent of maximum polymerization (Adapted from Heisler, et al., 2015).

polymerization assays. Polymerization of pyrene-labeled actin can be monitored by fluorescence methods because the incorporation of pyrene actin into an actin filament results in a greater than 7 fold increase in pyrene's fluorescence (Cooper, Walker, & Pollard, 1983). Initially, we tested the effects on spontaneous actin polymerization (in the absence of other polymerization-promoting proteins) and found that the oligomers only had a marginal effect on actin polymerization in the absence (Figure 8A) and presence of PFN1 (Figure 8B). In the absence of profilin, the mild acceleration of polymerization is likely do to the incorporation of a small amount of actin oligomers into the actin filament, which leads to the destabilization and severing of the filaments. In contrast, PFN1 precludes the incorporation of the oligomers into filaments and slows the overall rate of polymerization. However, the oligomers caused a potent inhibition of formin-mediated actin polymerization in the absence and presence of PFN1 (Figure 8 C, D). Measuring the tangent slope at 50% of maximum polymerization, and fitting it to an isotherm binding equation, we found inhibition constants that correlated with the results from single filament level experiments. The median inhibitory concentration of inhibition ( $IC_{50}$ ) was equal to  $2.0 \pm 0.2$  nM and  $4.8 \pm 0.6$  nM in the presence (Figure 9) and absence of PFN1 (additional data and equations can be found in figure 4E of Heisler et al, 2015).

We also tested the effect of ACD-crosslinked actin dimers, the only species of oligomers that can be purified to homogeneity, and found that they bind to and inhibit mDia1-mediated actin polymerization but to a lower extent (data can be found in supplementary figure 5, F to H of Heisler et al, 2015). This suggests that the inhibition of formins is propagated by the multivalent interaction of oligomers with formin homology domains.

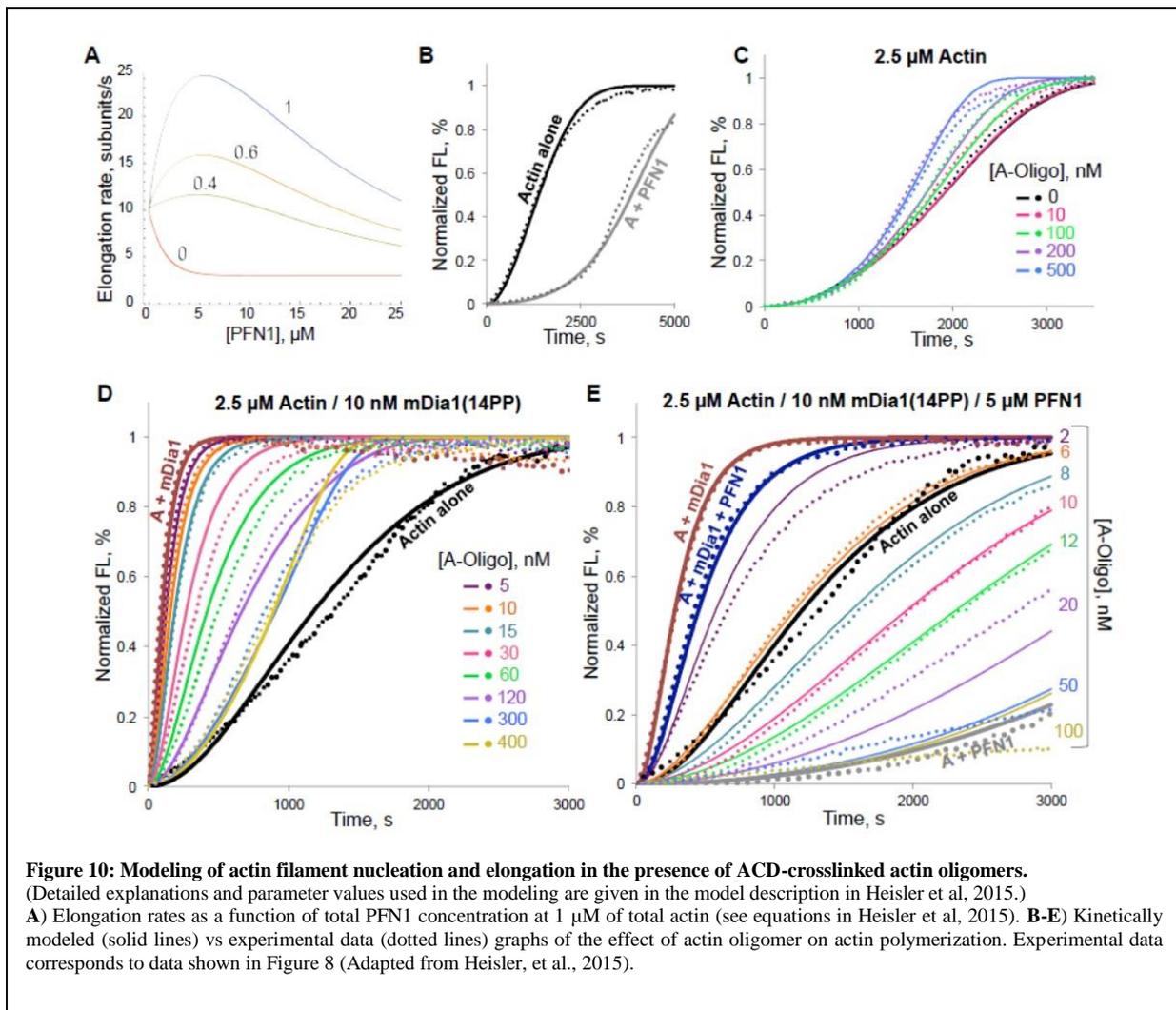
To elucidate which formin homology domain was responsible for the observed inhibition, we



generated truncation mutants of mDia1-FH1 domain (Figure 9). Shortening of the FH1 domain from 14 PP to 0PP (absence of an FH1 domain) reduced the inhibitory effects of the oligomers in the presence (Figure 9) and absence (data can be found in supplementary figure 6 of

Heisler et al, 2015) of PFN1. The  $IC_{50}$  values plateau at ~30 and ~16 nM for mDia1(FH2) constructs in the presence and absence of PFN1, respectively. This suggests that actin oligomers can bind to, and inhibit, the FH1 and FH2 domains of the formin family of proteins.

To understand the role of the FH1 and FH2 domains in the inhibition of formins by the oligomers, we utilized kinetic modeling based on the known rates of actin polymerization in the absence and presence of profilin and formins (Courtemanche & Pollard, 2013; Kovar, 2006) as well as the experimentally determined rates from our TIRF experiments (Figure 5). We found that our data fit a model of inhibition where the actin oligomers inhibit formin-mediated nucleation by binding to free formin with dissociation constants of 0.8 and 5 nM in the presence and absence of PFN1 (data can be found in supplementary figure 8, D and E of Heisler et al., 2015). Experimentally, the inhibition of nucleation in the absence of PFN1 was also observed in pyrenyl-actin (Figure 10, A and C) and TIRFM experiments (data can be found in supplementary figure 4,



F and G, and figure 7, G and H of Heisler et al., 2015). In the presence of profilin, nucleation by mDia1(14PP) cannot be separated from the rapid elongation of newly nucleated, mDia1(14PP)-controlled filaments, and therefore, we used mDia1(0PP) to monitor the inhibition mechanism (Figure 10, B and D). To improve the accuracy of our model, we accounted for the severing of filaments due to the incorporation of the actin oligomer in absence of profilin (Kudryashov, Durer, et al., 2008). In the presence of profilin, the actin oligomers are precluded from polymerization, and do not affect the stability of filaments.

Disruption of the actin cytoskeleton by altering host signaling pathways (e.g. Rho-GTPases (Lerm et al., 2000)) is not a new mechanism of toxicity for pathogenic bacteria (Aktories, Lang, Schwan, & Mannherz, 2011). However, we discovered a novel toxicity pathway by which ACD produces a new toxicity pathway. The de novo produced actin oligomers act as second messengers to target actin regulatory proteins. The unique combination of properties that the oligomers possess (Figure 2) bind with abnormally high affinity to formins, potently inhibiting both profilin dependent and independent manners. ACD takes host actin and converts it into toxic species with a disruptive gain-of-function property. Therefore, the previously understood mechanism of toxicity (passive accumulation of non-functional ACD-produced actin oligomers), is incomplete. Low concentration of the toxin can be efficient by taking a small amount of actin and converting it into secondary toxins that targets the low abundant, but essential protein, formins. This new finding of how an actin-specific toxin alters the cytoskeleton calls for a refreshed look at our understanding of the mechanism used by other actin altering toxins actually work.

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