

Elucidating Chiral Myosin-Induced Actin Dynamics: From Single Filament Behavior to Collective Structures

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Significance Statement

Myosins are essential motor proteins that drive intracellular transport, yet their ability to organize actin filaments into higher-order structures remains poorly understood. Here, we demonstrate that a plant myosin XI induces chiral curved motion of actin filaments and drives the spontaneous formation of stable, rotating actin rings through collective behavior. These structures, termed actin chiral rings (ACRs), emerge at actin concentrations approximating physiological conditions and show remarkable stability. Our findings uncover a previously unrecognized organizing principle of actin self-assembly driven by chiral myosin activity and provide a new framework for understanding how cytoskeletal chirality contributes to cellular organization across kingdoms.

Abstract

The myosin superfamily comprises over 70 classes, each with multiple subclasses, and shows substantial diversity in properties such as velocity, ATPase activity, duty ratio, and directionality. This functional diversity underpins the specialized roles of each myosin in various organisms, organs, and cell types. Recent studies have revealed that certain myosins induce chiral curved motions of actin filaments. However, this newly identified property remains largely unexplored. Here, we investigated this chiral motion *in vitro* using *Chara corallina* myosin XI (CcXI), which drives fast counterclockwise (CCW) movement of actin filaments. This chiral motion arises from asymmetric displacement at the filament's leading tip, and its curvature depends on the surface density of myosin. Surprisingly, at near-physiological actin concentrations, actin filaments exhibiting chiral curved motion undergo collective motion, spontaneously forming a novel structure—termed the actin chiral ring (ACR)—that exhibits persistent CCW rotation. ACRs display remarkable stability, continuing to rotate at their formation site until ATP is depleted, while maintaining their structure even after rotation ceases. This stability is unprecedented among reported collective motions of cytoskeletal proteins driven by various motors. Our findings demonstrate that myosins with chiral activity can autonomously organize actin filaments into stable, chiral structures through collective dynamics, providing new insights into actin self-organization by unconventional myosins. This study advances our understanding of the diverse functional roles of unconventional myosins and introduces a new paradigm for cytoskeletal organization.

Abbreviations: ACR, actin chiral ring; CcXI, *Chara corallina* myosin XI; MD, motor domain; SkII, skeletal muscle myosin II; CCW, counterclockwise; CW, clockwise; CbXI-3, *Chara braunii* myosin XI-3; SI, supplementary information.

Introduction

Phylogenetic analyses of myosin motor domain (MD) sequences have revealed remarkable diversity within the myosin superfamily. To date, at least 79 distinct classes have been identified (1). These myosin classes and subclasses exhibit broad functional diversity in properties such as actin sliding velocity, actin-activated ATPase activity, duty ratio (the fraction of ATPase cycles during which the motor domain remains strongly bound to actin), and directionality of actin filament movement (2). Recently, a novel functional property of myosin has been reported: rat myosin IC and *Drosophila* myosin ID drive actin filaments in a counterclockwise (CCW) trajectory, resulting in chiral curved motions (3, 4). This chiral activity is particularly noteworthy because the motor domain of *Drosophila* myosin ID plays a critical role in establishing left-right asymmetry (chirality) of *Drosophila* cells and organs (4-7). However, the mechanistic link between the chiral activity of myosin ID and the emergence of cellular or organismal chirality remains poorly understood.

The motility properties of each myosin isoforms, such as sliding velocity and the directionality of actin filament movement, have been extensively analyzed using *in vitro* motility assays. In this method, myosin molecules are randomly immobilized onto a coverslip, and fluorescently labeled actin filaments are introduced along with ATP to visualize their movements (8). Although the filaments move in various directions across the surface, their velocity and directionality are consistent with those observed for the corresponding myosin isoforms *in vivo*. Therefore, *in vitro* motility assays serve as a reliable method for quantifying the velocities and directionality of myosin-driven actin movements. These assays typically use fluorescent actin at a concentration of 3×10^{-4}

mg/ml, which is approximately 1/1,000 of the intracellular actin concentration (9). Such low concentrations are employed to allow for the clear observation of individual actin filament trajectories.

Recently, a modified *in vitro* motility assay has been developed to allow tracking of individual actin filaments under conditions that approximate intracellular actin concentrations. This improvement was achieved by supplementing the assay buffer with non-fluorescent actin at physiological levels, together with a low concentration of fluorescently labeled actin. Remarkably, under these dense actin conditions, actin filaments driven by skeletal muscle myosin no longer exhibited random motions. Instead, they displayed collective behaviors characterized by coordinated movement. These collective dynamics manifested in diverse forms, including polar clusters, nematic streams, and swirling vortices. A common structural feature among these diverse forms was the parallel alignment of actin filaments along their longitudinal axes (10).

To date, *in vitro* studies of myosin-driven collective motion of actin filaments have primarily employed skeletal muscle myosin, also known as conventional myosin. However, *in vivo*, skeletal muscle myosin is confined to the sarcomeres of muscle cells and does not participate in collective cytoskeletal dynamics. In most other cell types—including plant cells and non-muscle animal cells—the movement of actin filaments is predominantly driven by unconventional myosins. These intracellular actin movements often display a relatively ordered orientation rather than random trajectories. It is therefore plausible that unconventional myosins contribute to this ordered intracellular actin alignment through collective motion. Given that the motile properties of

unconventional myosins differ from those of skeletal muscle myosin, the resulting collective motion patterns are also likely to be distinct.

The collective behavior of the active matter is strongly governed by the geometric shape and motility characteristics of its components. For rod-shaped active matter, such as those composed of elongated filaments, collective motion is predominantly driven by lateral interactions, resulting in alignment along the longitudinal axes. This phenomenon has been observed in the collective dynamics of actin filaments driven by skeletal muscle myosin, where filaments behave as linear rods and exhibit motion aligned with their long axes, as described previously (10-14). Recently, increasing attention has been directed toward the collective behavior of chiral active matter, in which the components either possess intrinsic chirality or exhibit chiral motion patterns(15, 16). FtsZ, a bacterial homolog of the eukaryotic protein tubulin, polymerizes into filaments. When these filaments are anchored to a lipid surface via FtsA, they exhibit a treadmill-like motion and adopt a chiral shape. As the concentration of FtsZ increases, these filaments self-organize into rotating chiral rings through collective motion. It has been proposed that the formation of these chiral rings results from lateral interactions between moving FtsZ filaments with chiral geometry (15, 17).

In this study, we demonstrate that *CcXI* motor domain (MD) induces CCW actin movements. This chiral activity resembles that reported for rat myosin IC and *Drosophila* myosin ID (3, 4). However, unlike these class I myosins, *CcXI* MD exhibits fast motility (18-20), enabling detailed analysis within a shorter time window. We show that myosins with chiral activity, such as *CcXI* MD, possess a unique capacity to autonomously drive

the formation of highly stable, actin chiral structures—structures we term actin chiral rings (ACRs)—through collective motion of actin filaments. This finding provides new insights into the mechanisms of actin filament self-organization and expands our understanding of the broader functional roles of unconventional myosins in cytoskeletal dynamics.

Results

Chiral Curved Actin Motions by *CcXI*

In the standard *in vitro* motility assay (Fig. 1A), *CcXI* MD, a fast plant myosin XI(18-20), drove actin filaments in a counterclockwise (CCW) circular path when viewed from the actin side (clockwise (CW) when viewed from the objective lens side) (Fig. 1B: *CcXI* MD and Movie S1). Similar CCW movements have been previously reported for actin filaments driven by mouse myosin IC (3) and *Drosophila* myosin ID (4). The curvature of actin movements driven by *CcXI* MD was 17.0 ± 5.5 deg/ μm (Fig. 1C: *CcXI* MD), which exceeds the values reported for mouse myosin IC and *Drosophila* myosin ID using a similar assay (standard *in vitro* motility assay on glass surfaces) (3, 4). In contrast, actin filaments driven by rabbit skeletal muscle myosin II (SkII)—a widely used reference myosin—followed nearly straight trajectories, with an average curvature of 0.47 ± 8.8 deg/ μm (Fig. 1B and C: Rab SkII).

To determine whether this chiral activity is a common feature among class XI myosins, we measured the curvature of the actin trajectories using *CbXI-3* (*Chara braunii* myosin XI-3) MD, which belongs to the same class as *CcXI* (21). Although *CbXI-3* is a paralog of *CcXI* and shares high sequence similarity (98%) and identity (88%) with *CcXI*, the curvature of the actin trajectory induced by *CbXI-3* MD (3.1 ± 8.7 deg/ μm) was significantly smaller than that induced by *CcXI* MD. These results suggest that the curvature of the trajectory is determined by subtle variations in the myosin amino acid sequence, rather than by myosin class. This finding is consistent with previous reports indicating that only a subset of class I myosins generate curved actin trajectories whereas most do not (3, 4).

Characterization of Chiral Curved Actin Motions

To investigate how CcXI-induced actin filament curvature depends on myosin density, we performed a series of *in vitro* motility assays using chambers with a spacer thickness of 0.24 mm. CcXI MD was immobilized onto the coverslip surface, and increasing the concentration of myosin introduced into the chamber resulted in a progressive increase in actin filament curvature, which was observed up to a concentration of 0.5 μ M (Fig. 1D). At this concentration, the surface myosin density was estimated to be approximately 3.6 molecules per 10×10 nm² area. Given that the diameter of the myosin XI MD is approximately 4 nm (21), this density likely corresponds to near-saturation of the coverslip surface. Above this concentration, no further increase in curvature was detected, suggesting that the effect plateaued due to saturation of available myosin-binding sites. These results indicate that actin filament curvature increases in proportion to surface myosin density until saturation is reached.

To elucidate the mechanism underlying this chiral curved motion, we tracked both the leading and trailing ends of individual actin filaments. We found that both ends followed the same trajectory (Fig. 1E), indicating that the curved motion is primarily generated by asymmetric displacement at the filament's leading tip, while the remainder of the filament passively follows the path without actively contributing to the curvature.

Emergence of ACR through Collective Motion of Chiral Curved Actin

Traditionally, standard *in vitro* motility assays have been conducted using actin concentrations far below physiological levels, typically around 0.0005 mg/ml (9). These

low concentrations are preferred because higher concentrations of fluorescently labeled actin can obscure individual filaments, making it difficult to observe their movement. However, recent advances have enabled assays to be performed at actin concentrations closer to physiological levels (~1 mg/ml) by combining a low concentrations of fluorescently labeled actin with non-fluorescently labeled actin at physiological levels.

Using this approach, studies with skeletal muscle myosin have revealed that collective motion emerges even when myosin and actin filaments were randomly distributed on the coverslip. Remarkably, under these conditions, actin filaments exhibited highly organized collective motion, characterized by parallel filament arrays. Moreover, most filaments within these aligned arrays moved in the same direction, suggesting that collective motion not only facilitates parallel alignment of actin filaments but also promotes directional uniformity (10, 11, 14, 22-25).

Given that skeletal muscle myosin drives actin filaments along straight trajectories parallel to their longitudinal axes, whereas *CcXI* induces chiral curved motion, we hypothesized that applying this modified motility assay with *CcXI* under actin concentrations approximating physiological conditions might result in a different type of collective motion. To test this hypothesis, we performed the modified motility assay using *CcXI* MD. We found that collective motion emerged effectively at initial actin concentrations between 0.1 and 0.3 mg/ml, with the strongest effects at 0.1 mg/ml. Therefore, subsequent experiments were conducted under this condition to maximize emergence of collective behavior.

The assay was initiated by adding a buffer containing 0.1 mg/ml actin (without ATP) to a chamber precoated with *CcXI* MD. After a 10-min incubation, an ATP-containing assay buffer (without actin) was introduced to remove unbound filaments, and imaging was initiated. Under these conditions, the actin filament density reached approximately 15 ± 4.1 filaments/ μm^2 (*SI Appendix*, Fig. S1), consistent with values reported in previous studies of skeletal muscle myosin II-driven collective actin motion (23).

Immediately after the ATP-containing buffer was added to the chamber, the *CcXI*-driven actin filaments moved randomly. However, after approximately 20 min, they self-organized into a ring-like structure, in which the entire ring exhibited collective motion, rotating predominantly in the CCW direction when viewed from the actin side. Notably, this rotational direction matched the trajectories observed for individual actin filaments at lower actin concentrations. We designated this structure the actin chiral ring (ACR) (Fig. 2A, 2B and Movie S2). The formation of ACRs was confirmed even when Cy3-labeled actin was used instead of rhodamine-phalloidin-labeled actin, ruling out the possibility that ACR formation was an artifact of phalloidin labeling.

The dynamic process of ACR formation was continuously monitored, revealing distinct intermediate stages. Although image clarity was partially compromised by photobleaching due to the prolonged light exposure, key structural transitions were still observable. Initially, actin filaments exhibited parallel stream-like flow that gradually coalesced into nascent ring-like structures. These nascent rings subsequently developed into fully formed ACRs, with their width increasing over time until reaching a stable state (Movie S3 and Fig. 2C: *CcXI*).

Structural Characterization of ACRs

We analyzed the structural characteristics of the ACRs. The ACRs exhibited limited size variation, indicating a high degree of structural uniformity. The outer and inner diameters were $21.8 \pm 2.4 \mu\text{m}$ and $11.0 \pm 2.3 \mu\text{m}$, respectively (Fig. 3A). The inner curvature, calculated from these inner diameters, was $10.0 \pm 2.3 \text{ deg}/\mu\text{m}$ (Fig. 3A: IC). Although this value was slightly smaller than the curvature of individual actin filaments in the absence of collective motion ($17.0 \pm 5.5 \text{ deg}/\mu\text{m}$, Fig. 1C), it closely matched it in magnitude. This similarity, along with the consistent CCW direction of rotation, suggests that CcXI-induced curved actin motions are critical for ACR formation, although the curvature appears to be slightly attenuated during collective motion.

In contrast, the outer curvature of the ACRs, calculated from the outer diameters, was $5.3 \pm 0.6 \text{ deg}/\mu\text{m}$ (Fig. 3A: OC), significantly lower than that of individual actin filaments. This reduction in outer curvature likely imposes a physical size limit on the ACRs, contributing to their nearly uniform dimensions. Furthermore, the consistent size of ACRs below a certain threshold suggests that filament–filament affinity does not play a significant role in their formation. If ACR formation were driven by filament affinity, the structures would be expected to grow continuously over time without exhibiting a defined size limit.

The ACRs exhibited remarkable temporal and spatial stability. Once formed, they continued to rotate at the same location without positional displacement (Fig. 2B and Movie S2). When external fluid flow was intentionally applied after formation, the ACRs

maintained their structure and continued to rotate at the same location, highlighting their structural integrity and robustness. Moreover, the ACRs preserved their ring-shaped architecture even after rotational motion ceased due to ATP depletion. Taken together, these observations underscore the exceptional stability of the ACR as a structural unit.

To analyze the movement of individual actin filaments within the ACR, we adjusted the ratio of Cy3-labeled to non-fluorescent actin (*SI Appendix*, Supplementary Materials and Methods) to enable clear visualization of each filament. Our observations revealed that a substantial majority (88%) of actin filaments within the ACR rotated in the CCW direction, while a minority (12%) exhibited CW rotation (Fig. 3B). This result is somewhat surprising, given that the entire ACR appears to rotate in the CCW direction (Movie S2), consistent with the observation that *CcXI* drives individual filaments in a CCW direction (Fig. 1B and C). The CW-rotating filaments likely represent those that entered the ACR from the opposite direction and continued to move without reorienting, as discussed below. The observation that 88% of filaments within the ACR exhibit CCW motion reflects a strong bias toward unidirectional alignment. This proportion is notably higher than the 60–80% directional alignment during the collective motion of parallel actin filaments induced by skeletal muscle myosin (23). These results suggest that ACR formation facilitates more efficient unidirectional alignment of actin filaments compared to conventional collective motion systems.

To determine the number of actin filaments per ring width within the ACR, we employed the same approach of adjusting the ratio of Cy3-labeled to non-fluorescent actin (*SI Appendix*, Fig. S2). This method enabled us to estimate the number of filaments per ring

width, revealing an average of 320 ± 10 filaments and an estimated interfilament spacing of approximately 22 ± 9.8 nm (Fig. 3C). This spacing—approximately three times the diameter of a single actin filament—suggests that filaments within the ACR are relatively spaced rather than densely packed. Notably, this interfilament distance is consistent with the spacing (~ 20 nm) reported for collective actin motion driven by skeletal muscle myosin in previous studies (25).

To investigate the dynamics of chiral curved actin filaments interacting with the ACR, we again employed the method of adjusting the ratio of Cy3-labeled to non-fluorescent actin. This approach enabled detailed observation of collision outcomes. When actin filaments approached the ACR from the the direction opposite to its rotation, 80% were repelled, 10% were integrated into the ACR and began rotating in the CCW direction, and the remaining 10% were incorporated but rotated in the CW direction (Fig. 3D, right). This latter group likely represents the minority CW-rotating population observed within the ACR, as noted above. In contrast, when actin filaments approached from the same direction as the ACR's rotation, 90% were assimilated and adopted CCW rotation, while the remaining 10% were excluded (Fig. 3D, left).

Effects of Actin Bundling Protein and Methylcellulose on ACR Formation

In the cellular environment, actin-binding proteins (ABPs) play an essential role in modulating actin networks by bundling actin filaments. To investigate the influence of ABPs on ACR formation, we focused on villin, a well-characterized actin-bundling protein. In modified *in vitro* motility assays under high actin concentrations, the presence of villin markedly accelerated ACR formation, reducing the time required from

approximately 20 min (in the absence of villin) to just a few minutes. The ACRs formed in the presence of villin were significantly smaller, with an outer diameter of approximately 5 μm —about quarter of the ~ 20 μm diameter observed without villin. Notably, these smaller ACRs formed in the presence of villin maintained rotational behavior at their formation site (Fig. 4A, Movie S4), similar to the ACRs formed in the absence of villin. This observation suggests that villin-mediated size reduction does not impair either the rotational stability or the self-organization process of ACRs.

Methylcellulose, a polymer widely used in *in vitro* studies to mimic intracellular molecular crowding (26, 27), is known to promote actin bundling through the depletion effect (28, 29). The presence of methylcellulose markedly altered ACR formation, leading to the emergence of large, vortex-like ACRs with outer diameters reaching up to 100 μm —substantially larger than the ~ 20 μm diameter observed in its absence. These vortex-like ACRs exhibited the same CCW rotation as those formed without methylcellulose (when viewed from the actin side). However, they were unstable and rapidly disintegrated after formation (Fig. 4B, Movie S5).

We next examined the combined effects of villin and methylcellulose on ACR formation. In the presence of both factors, ACRs exhibited slightly larger diameters—approximately 30 μm —compared to the typical ~ 20 μm ACRs. The direction of rotation of these ACRs was CCW, which was consistent with the direction observed under other conditions. However, similar to the vortex-like ACRs formed in the presence of methylcellulose alone, they were less stable and more prone to disintegration (Fig. 4C, Movie S6). These results suggest that while villin accelerates ACR formation and reduces its size, methylcellulose

331 promotes the formation of larger and less stable ACR structures. Together, these two
332 factors synergistically influence the size and stability of ACRs.

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Discussion

In this study, we demonstrated that *CcXI* drives actin filaments in the CCW direction and that, at actin concentrations comparable to those found in cells, *CcXI* induces the autonomous formation of cell-sized ACRs through collective motion. Remarkably, ACRs are exceptionally stable structures, maintaining continuous rotation at their formation site until ATP depletion. Furthermore, even after ATP depletion, these structures preserved their integrity, underscoring their robustness and stability as structural units.

Insights into the Mechanism of CCW Actin Movement

While most myosins move actin filaments along the filament axis, recent studies have shown that certain class I myosins induce chiral curved motion, driving actin filaments in a CCW direction (3, 4). However, the underlying mechanism of this motion remains poorly understood, partly due to the extremely slow motility of class I myosins. To investigate the mechanism of myosin-induced curved motion, we conducted a detailed analysis of the CCW motion driven by *CcXI*, a fast-moving myosin XI (18-20, 30, 31)(32), which allowed for thorough analysis within a shorter time window. Our analysis revealed that the trailing end of the filament followed the same path as the leading end (Fig. 1E), indicating that the critical displacement for chiral curvature occurs predominantly at the filament tip. The tip of the actin filament, characterized by its high degree of freedom, is readily bent by oblique forces relative to the filament axis, likely generated by the power stroke of *CcXI*, as discussed below. In contrast, bending of other regions along the filament is restricted by the strong, rigor-state binding of *CcXI*, which anchors the filament at both anterior and posterior contact points in these regions (*SI Appendix*, Fig. S3).

Our study reveals a positive correlation between the curvature of actin filament trajectories and surface myosin density. This finding contrasts with the well-established inverse relationship between actin velocity and myosin density, in which increased myosin density typically reduces actin filament velocity. This velocity reduction is thought to result from strongly bound myosin molecules hindering the progression of filaments driven by other myosins performing power strokes (33, 34). In contrast, the observed increase in actin filament curvature with higher myosin density likely originates at the filament tips, where a higher myosin density induces more frequent bending events (Fig. 1E, *SI Appendix*, Fig. S4). As shown in Fig. S4, this tip-specific bending frequency scales with local myosin density, highlighting a mechanistic distinction from the velocity–density relationship.

Why does the leading tip of *CcXI*-driven actin filaments move obliquely to the filament axis? To address this question, we must reconsider the mechanism by which myosin drives actin filaments. The lever-arm model, which is currently the most widely accepted explanation for myosin-driven actin movement, proposes that actin filament displacement is driven by a swinging motion of the lever arm. In this model, the lever arm undergoes a power stroke (swinging motion) relative to the actin filament axis, thereby propelling the filament forward (35). Myosins such as *CcXI*, mouse myosin IC, and *Drosophila* myosin ID, which induce CCW actin motion, may achieve this by swinging their lever arms at an oblique angle relative to the actin filament axis. To test this hypothesis, we compared the rigor-state structures of acto-*CcXI* MD, resolved by cryo-electron microscopy (32), with that of acto-SkII S1, also determined by cryo-electron microscopy (36). We initially

hypothesized that the lever-arm position of *CcXI* in the rigor state would be oblique to the actin filament. However, structural analysis revealed that the lever arm positions of *CcXI* and *SkII* in the rigor state were nearly identical, both aligning parallel to the actin filament (*SI Appendix*, Fig. S5). Thus, the post-power stroke conformation alone cannot account for the CCW actin movements driven by *CcXI*.

In contrast, in the pre-power stroke state, *CcXI* may adopt a lever-arm orientation that deviates from the filament axis. This oblique configuration could result in a power stroke that is inclined rather than parallel to the filament axis, thereby generating chiral curved actin motion. A recent cryo-electron microscopy study of mouse myosin IC in both the ADP-bound and the rigor states provides structural evidence supporting this hypothesis (37). This study demonstrated that in the ADP-bound state, which corresponds to the pre-power stroke state, mouse myosin IC exhibits a tilted lever arm relative to the filament axis. In contrast, in the rigor state, which corresponds to the post-power stroke, the lever arm is nearly parallel to the filament axis. These findings suggest that an oblique power stroke, as observed in mouse myosin IC, is responsible for the generation of chiral curved actin motion. Similarly, *CcXI* may also adopt a pre-power stroke lever-arm orientation that deviates from the filament axis, thereby producing an oblique power stroke and subsequently inducing chiral curved motion.

Mechanism of ACR Formation

Under high actin concentration, actin filaments undergoing chiral curved motion driven by *CcXI* self-organized into ACRs through collective motion (Fig. 2A and B, Movies S2). To understand how the ACRs are formed, it is instructive to apply insights from the well-

studied mechanism of SKII-driven collective motion to the context of *CcXI*-driven collective motion. Under similar high actin conditions, SKII induces the formation of organized structures such as polar clusters, nematic streams, and swirling vortices (11, 12, 22-25). In these SKII-induced structures, the filaments are aligned parallel to one another. This arrangement likely reflects the liquid crystalline properties of actin filaments: when freely interacting at high densities, straight actin filaments behave as rod-like macromolecules that naturally align along their longitudinal axes to minimize free energy (Fig. 2C: SkII) (38, 39). Because of the differences in interaction effects between chiral curved and straight actin filaments, it is not surprising that the collective motion of actin filaments induced by *CcXI* differs from that induced by SKII. Based on the liquid crystalline properties of actin filaments, we propose that chiral curved actin filaments driven by *CcXI* initially align with the same polarity (Fig. 2C: *CcXI*, middle), and subsequently accumulate to form ACRs (Fig. 2C: *CcXI*, right). Notably, the filament behavior observed during ACR formation (Movie S3) closely resembles the schematic model shown in Figure 2C (*CcXI*).

Parallel streaming of actin filaments induced by SkII has been proposed to result from orientation changes during filament collisions. However, previous studies have shown that the angular adjustments during two-filament collisions are insufficient to account for this phenomenon, indicating that interactions among multiple filaments are necessary to establish parallel streaming (24, 25). Consistent with these findings, we also did not observe significant angle changes in orientation during collisions between two chiral curved actin filaments, similar to the case with straight filaments (24). These observations suggest that effective alignment, whether in straight or chiral curved filaments, requires

multifilament interactions rather than simple binary collisions.

To examine the role of multifilament interactions, we simulated multifilament collisions using a simplified model, where the orientation of the leading edge of each filament adapted to that of neighboring filaments upon collision. Simulations with straight actin filaments resulted in bidirectional (nematic) streaming, rather than unidirectional (polar) streaming (Movie S7). In contrast, simulations with chiral curved actin filaments led to the emergence of curved polar arrays that self-organized into ACR-like structures (Movie S8), closely resembling the experimentally observed ACR formation process (Movie S3). The finding that polar orientation was more likely to emerge in simulations of chiral curved actin filaments is consistent with our experimental observations, where the collective motion of chiral curved actin filaments showed a significantly higher percentage of polar orientation (88%) compared with that of straight actin filaments (60-80%) (23) (Fig. 3B).

Collective motion of chiral curved actin filaments exhibits significantly greater structural uniformity and stability compared with that of straight actin filaments. In previous studies, straight actin filaments exhibited collective motions in various forms, such as waves and clusters, with variations in persistence and directionality (22-25). In contrast, chiral curved actin filaments consistently self-organized into ACRs, which invariably rotated in the CCW direction. This remarkable structural and directional consistency is likely a key factor contributing to the long-term stability of ACRs.

Similar self-organized chiral rings are also observed in the bacterial cytoskeletal system.

FtsZ, a bacterial tubulin homolog, polymerizes into curved filaments that exhibit chiral treadmilling motion when anchored to lipid membranes via FtsA. At sufficiently high concentrations, these curved filaments self-organize into chiral rotating rings *in vitro* (17, 40). Brownian dynamics simulations and coarse-grained modeling have further supported the autonomous formation of these structures (15, 40). FtsZ plays a central role in bacterial cytokinesis by assembling into the Z-ring at the future division site. These findings suggest that the capacity of cytoskeletal proteins to form chiral rings through collective motion may reflect a fundamental organizing principle of cytoskeletal systems. Moreover, the formation of such chiral structures by cytoskeletal elements with intrinsic chirality may represent a widespread and evolutionarily conserved mechanism across diverse organisms.

Functions of ACR in Cellular Dynamics

ACR-like structures, referred to as acquosomes, have been identified in various plant cell types, including *Arabidopsis*, tobacco BY2 cells, zinnia, and *Characean* algae. When cytoplasmic extracts from *Characean* algae were observed under a dark-field microscope, acquosomes exhibited rotational motion on the glass surface in the same direction as the ACRs characterized in this study. Furthermore, negative-staining electron microscopy revealed that these structures are composed of actin filament bundles with uniform polarity (41). These findings suggest that the acquosomes formed *in vivo* possess structural and dynamic properties analogous to those of ACRs reconstituted in this study under *in vitro* conditions.

Acquosome formation has been reported to occur transiently during specific cellular

events such as the onset of growth or in response to injury, and this process depends on myosin activity. In the case of pollen tube cells, for instance, acquosomes appear briefly before the initiation of elongation and are subsequently reorganized into polarized actin bundles that guide directional tip growth (42, 43)(44-46). Such polarized bundles are essential for unidirectional cytoplasmic streaming, a fundamental mechanism for intracellular transport in plant cells. Previous studies have demonstrated that these structures can form autonomously through interactions between actin filaments and myosin XI (47, 48), and that actin-bundling proteins such as plant villin facilitate their formation by crosslinking filaments with uniform polarity (49). Our current findings indicate that similarly polarized bundles can also form via ACRs, which emerge from the collective motion of actin filaments driven by myosin with chiral activity, *CcXI* (Fig. 2B and Supplementary Movies 2 and 3). Moreover, the presence of villin significantly accelerates this process (Fig. 4A, C and Supplementary Movies 4 and 6). Integrating our findings with previous observations, we propose that ACRs may serve as functional equivalents of acquosomes in organizing polarized actin structures, with villin acting as a potential enhancer of this process *in vivo*.

Drosophila myosin ID, like *CcXI*, drives the chiral curved motion of actin filaments (4). It also plays a crucial role in establishing cellular and organ-level chirality (4-7), although the underlying mechanism remains poorly understood. Given that ACRs are cytoskeletal assemblies exhibiting chirality at the cellular scale, their formation by *Drosophila* myosin ID could provide key insights into the mechanism by which myosin ID induces cell chirality. Therefore, it is essential to determine whether *Drosophila* myosin ID can induce ACR formation under physiological actin concentrations, as observed for *CcXI*.

Although the functions of myosins have been extensively studied, little attention has been paid to their chiral activity, potentially leading to the oversight of myosins possessing this property. One reason for this oversight is that the chiral activity depends on myosin density and requires a sufficiently high concentration of myosin molecules, as demonstrated in Figure 1D. However, conventional *in vitro* motility assays are typically performed at low myosin densities to ensure smooth actin filament movement, thereby making the detection of chiral activity difficult. Consequently, despite numerous studies on CcXI since 2003 (18-20, 30-32), its chiral activity has remained unidentified until the present study.

Uncovering the chiral activity of myosins opens new avenues for understanding how these motors regulate intracellular actin dynamics. Our findings demonstrate that myosin chiral activity can drive the autonomous formation of stable, highly ordered actin architectures, revealing an additional layer of complexity in cytoskeletal organization. This discovery broadens the functional landscape of myosins beyond conventional attributes such as velocity, ATPase activity, duty ratio, and directionality. Further exploration of myosin chiral activity in cellular contexts, including cytoskeletal organization and cell chirality, may provide key insights into its role in shaping cytoskeletal architecture and coordinating cellular organization.

Materials and methods (see also *SI Appendix, Supplementary Materials and Methods*)

Protein expression, and purification

CcXI MD and *CbXI-3* MD were expressed in insect cells (High Five™, Life Technologies) and purified using nickel-affinity and FLAG-affinity resins as previously described (20, 21). Further details are described in the *SI Appendix, Materials and Methods*. Skeletal muscle myosin II was purified from rabbits as described previously (50, 51). Skeletal muscle actin was purified from Chicken scapes using the method of Spudich and Watt (52). Cy3-labeled skeletal muscle actin was prepared as described previously (51, 53).

Standard *in vitro* motility assay

The standard *in vitro* motility assay was performed as previously described (20, 21). Briefly, 0.2 mg/ml of anti-c-Myc antibody was perfused into the motility chamber. After washing with assay buffer, 0.15 mg/ml of myosin molecules with a c-Myc epitope tag were perfused into the chamber to immobilize the myosin molecules on the coverslip surface using an anti-c-Myc antibody. Next, the assay chamber was infused with either assay buffer containing 0.0003 mg/ml rhodamine-phalloidin-labeled actin or the same concentration of phalloidin-labeled Cy3-actin. The composition of the assay buffer was as follows: 25 mM Hepes-KOH (pH 7.4), 25 mM KCl, 4 mM MgCl₂, 3 mM ATP, 10 mM DTT, and an oxygen scavenging system (120 µg/ml glucose oxidase, 12.8 mM glucose, and 20 µg/ml catalase). Observations of motility were conducted at 25°C.

Modified *in vitro* motility assay at high actin concentration

Myosin molecules were immobilized on the coverslip using an anti-c-Myc antibody as described in the standard *in vitro* motility assay (19, 20)(21, 30, 54). The assay chamber was subsequently infused with assay buffer without ATP, either containing 0.005–0.01 mg/ml rhodamine-phalloidin-labeled actin and 0.1 mg/ml actin or assay buffer containing 0.005 mg/ml phalloidin-labeled Cy3- labeled actin and 0.1 mg/ml actin. The actin used was pre-treated with gelsolin (Sigma-Aldrich, product number: G8032) at a molar ratio of 1000:1 to actin in the presence of 1 mM CaCl_2 , resulting in an average length of approximately 5 μm . It should be noted that the average length of actin filaments became shorter due to severing events occurring during movement. After 10 min of incubation, the assay buffer with ATP was infused to remove unbound filaments and observation began. Cy3-labeled actin was prepared by labeling actin with Cy3-succinimide. To avoid motility artifacts, the proportion of Cy3-labeled actin molecules within the filaments was kept below 10%, as previously reported (51). The composition of the assay buffer was the same as that used for the standard *in vitro* motility assay: 25 mM Hepes-KOH (pH 7.4), 25 mM KCl, 4 mM MgCl_2 , 3 mM ATP, 10 mM DTT, and an oxygen scavenging system (120 $\mu\text{g/ml}$ glucose oxidase, 12.8 mM glucose, and 20 $\mu\text{g/ml}$ catalase) . In some experiments, the concentration of KCl in the assay buffer was 150 mM. Observations of motility were conducted at 25°C.

Measuring the curvature of the trajectory of a single actin filament in the standard *in vitro* motility assay

An orientation difference-based method was used to calculate curvature from a set of points. Specifically, the curvature was calculated by dividing the orientation angle

differences by the distance between two points using the orientation information of the point sequence (55). The X and Y coordinates of the leading end of a single actin filament were measured at 1 μm intervals over 21 points using Bohbo Soft (56). We then determined the curvature of the trajectory of a single actin filament from a set of 21 points spaced 1 μm apart by dividing the total angular variation by the total distance of movement across these 21 points. For each myosin type studied, curvature measurements were taken from approximately 30 actin filaments and then averaged to obtain the final values.

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Figure legends

Figure 1. Chiral curved motion of actin filaments driven by CcXI in the standard *in vitro* motility assay. (A) Schematic diagram showing the standard *in vitro* motility assay. Myosin molecules were randomly immobilized on a coverslip, and fluorescently labeled actin filaments are added along with ATP to visualize filament motion. (B) Representative trajectories of actin filaments over a 5-second interval driven by *Chara corallina* myosin XI MD (CcXI MD) and rabbit skeletal muscle myosin II (Rab SKII). The color gradient from red to purple indicates the progression of time from 0 to 5 seconds. (C) Histogram showing the distribution of actin filament curvatures in a standard *in vitro* motility assay with CcXI MD and Rab SKII. Curvature (degrees per micrometer) was calculated from orientation changes at 1 μ m intervals over 21 points along individual filaments. Positive

curvature values indicate CCW movement when viewed from the actin side. Each distribution represents measurements from 30 actin filaments per myosin condition. (see *SI Appendix*, Supplementary Materials and Methods). (D) Dependence of filament curvature on surface myosin concentration in a standard *in vitro* motility assay with CcXI MD. The x-axis represents the myosin concentration (μM) applied to a chamber with a spacer thickness of 0.24 mm. The y-axis shows the mean filament curvature $\pm$ standard deviation ($n = 27\text{--}30$ filaments per condition). (E) Overlay of the trajectories of the leading (red) and the trailing (blue) ends of a single actin filament in a standard *in vitro* motility assay with CcXI MD. The trailing end follows the same path as the leading end, indicating that curvature is generated primarily at the filament tip and is maintained along its length.

Figure 2. Autonomous formation of ACRs through the collective motion of chiral curved actin filaments driven by CcXI in the modified *in vitro* motility assay. (A)

Schematic illustration of ACR formation under conditions with elevated actin filament density. ACRs emerged within approximately 20 min after ATP addition, exhibiting CCW rotation when viewed from the actin side and CW rotation when viewed from the lens side. (B) Fluorescence microscopic images of fully formed ACRs. Once formed, the ACRs rotated continuously in the CCW direction at the same location where they initially developed.. The corresponding video is provided as Supplementary Movie 2. (C) Schematic representation of the formation process of collective actin motion under elevated actin filament density. **CcXI:** Upon ATP addition, individual actin filaments driven by CcXI initially exhibited CCW chiral curved trajectories and progressively aligned into a parallel stream-like collective motion. These filaments then gradually assembled into ring-shaped structures, which subsequently rotated in the CCW direction. **SKII:** For comparison, a schematic of the collective motion of actin filaments driven by skeletal muscle myosin II (SkII), as reported in previous studies, is shown. In contrast to

CcXI, SkII drives straight actin filaments that form parallel streams without ring formation.

Figure 3. Analysis of actin filament motion within ACR structures. (A) Quantification of the outer and inner diameters of the ACRs and the width of the ring. The outer and inner diameters were measured by fitting circles using ImageJ. The data represent the mean $\pm$ standard deviation of 120 ACRs. Except for actin concentration, all assay conditions were identical to those used in the standard *in vitro* motility assay for measuring single-filament curvature, including a solution containing 25 mM Hepes-KOH (pH 7.4), 25 mM KCl, 4 mM MgCl₂, 3 mM ATP, and 10 mM DTT. (B) Directional analysis of 90 actin filaments (AFs) from six ACR rings revealed that 79 AFs (88%) moved in CCW direction, while 11 AFs (12%) moved in CW direction. This analysis was conducted by reducing the ratio of Cy3-labeled actin, as described in the SI *Appendix, Supplementary Materials and Methods*. (C) The filament density within ACRs was estimated by lowering the proportion of Cy3-labeled actin, as illustrated in Fig. S2. Across 20 ACRs, the number of filaments per ring width averaged 320 ± 20 , with an inter-filament spacing of approximately 22 ± 9.8 nm. These values indicate relatively sparse filament packing within the ring structure. (D) Outcomes of filament-ACR collisions depending on the approach direction. (Left) When AFs approached in the same direction as ACR rotation, 90% were assimilated into the ACR and began rotating CCW, while 10% are ejected. (Right) When AFs approached from the opposite direction of ACR rotation, 80% are repelled, 10% were incorporated and rotated CCW, and 10% were incorporated but rotated CW. The latter population likely corresponds to the minority CW-rotating filaments observed in (B). Each category was quantified based on observations of 30 AFs for each direction.

Figure 4. Effects of Actin Bundling Protein and Methylcellulose on ACR Formation

(A) Formation of ACRs in the presence of villin. To assess the impact of actin-bundling proteins on ACR formation, 1.5 μM villin was added to the modified *in vitro* motility assay. Within 1 to 2 min after the start of the reaction, numerous small ACRs with outer diameters of approximately 5 μm appeared, which were rotating in CCW direction. These smaller ACRs exhibited robust and continuous CCW rotation at their formation sites, resembling the behavior of ACRs formed without villin. The video corresponding to Fig. 4(A) is available as Supplementary Movie 4, illustrating the dynamic process shown in this figure. (B) Formation of ACRs in the presence of methylcellulose. To mimic intracellular molecular crowding, 0.5% methylcellulose was included in the assay. This condition led to the emergence of large, vortex-like ACRs with outer diameters of approximately 100 μm appeared. These large, vortex-like ACRs rotated in CCW direction, similar to ACRs formed in the absence of methylcellulose. However, unlike ACRs formed without methylcellulose, these structures were unstable and disintegrated shortly after formation. The video corresponding to Fig. 4(B) is available as Supplementary Movie 5, illustrating the dynamic process shown in this figure. (C) Formation of ACRs in the combined presence of villin and methylcellulose. When both 1.5 μM villin and 0.5% methylcellulose were added, the resulting ACRs exhibited intermediate diameters of approximately 60 μm . Similar to other conditions, these ACRs rotated in CCW direction. However, as observed with methylcellulose alone, these large ACRs were unstable and rapidly disintegrated shortly after formation. The video corresponding to Fig. 4(C) is available as Supplementary Movie 6, illustrating the dynamic process shown in this figure.

Fig. 1

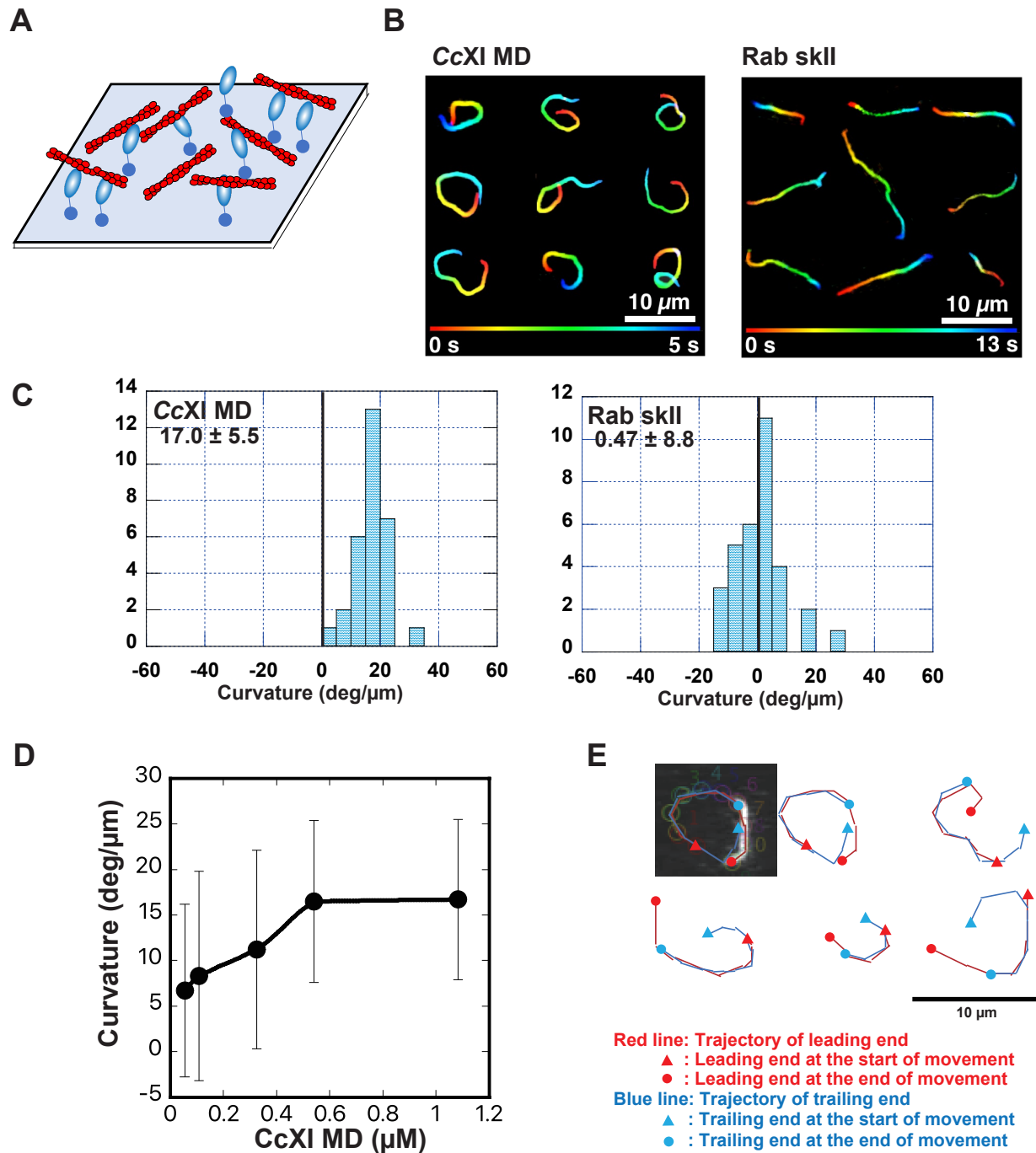


Fig. 2

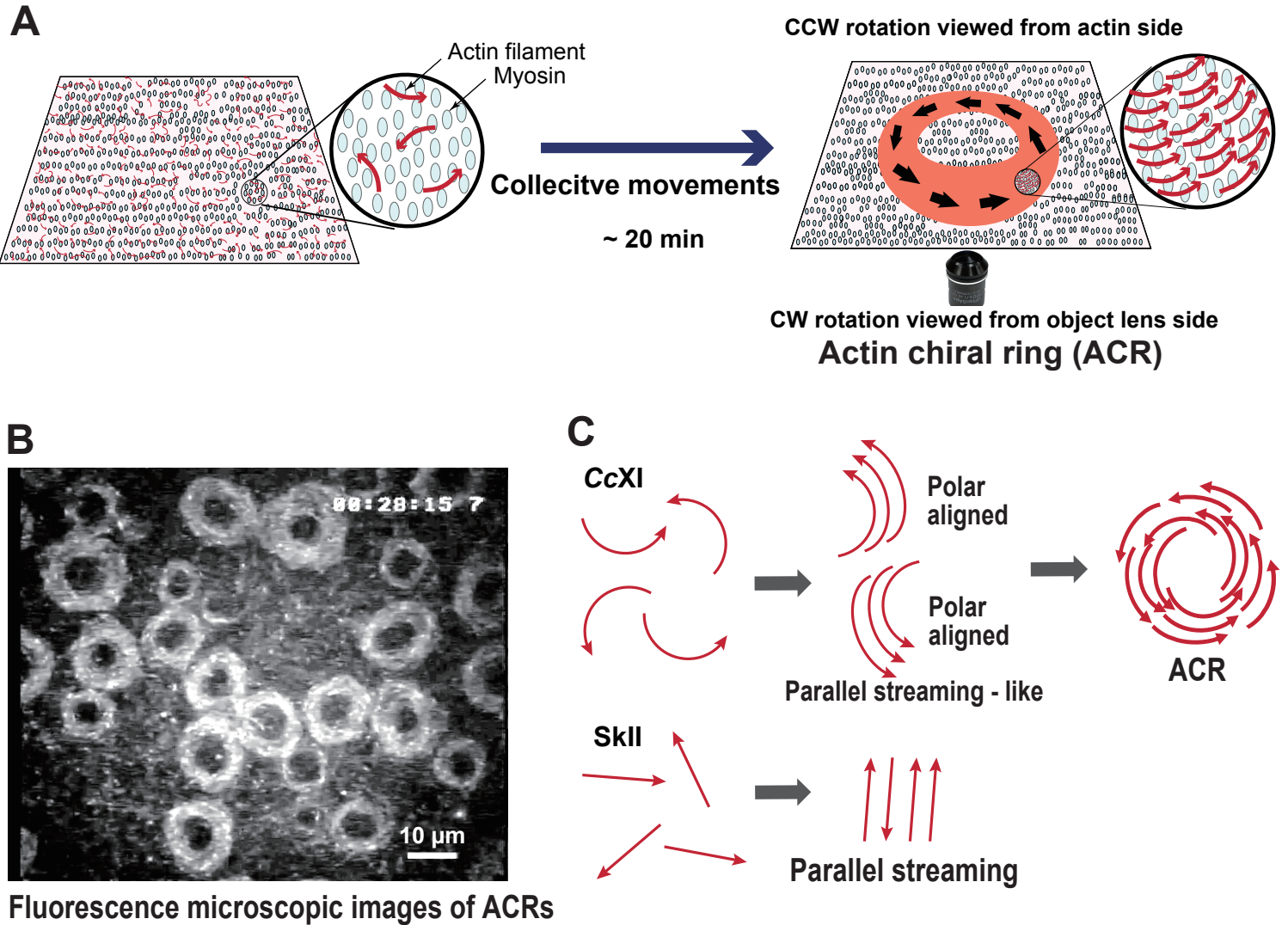
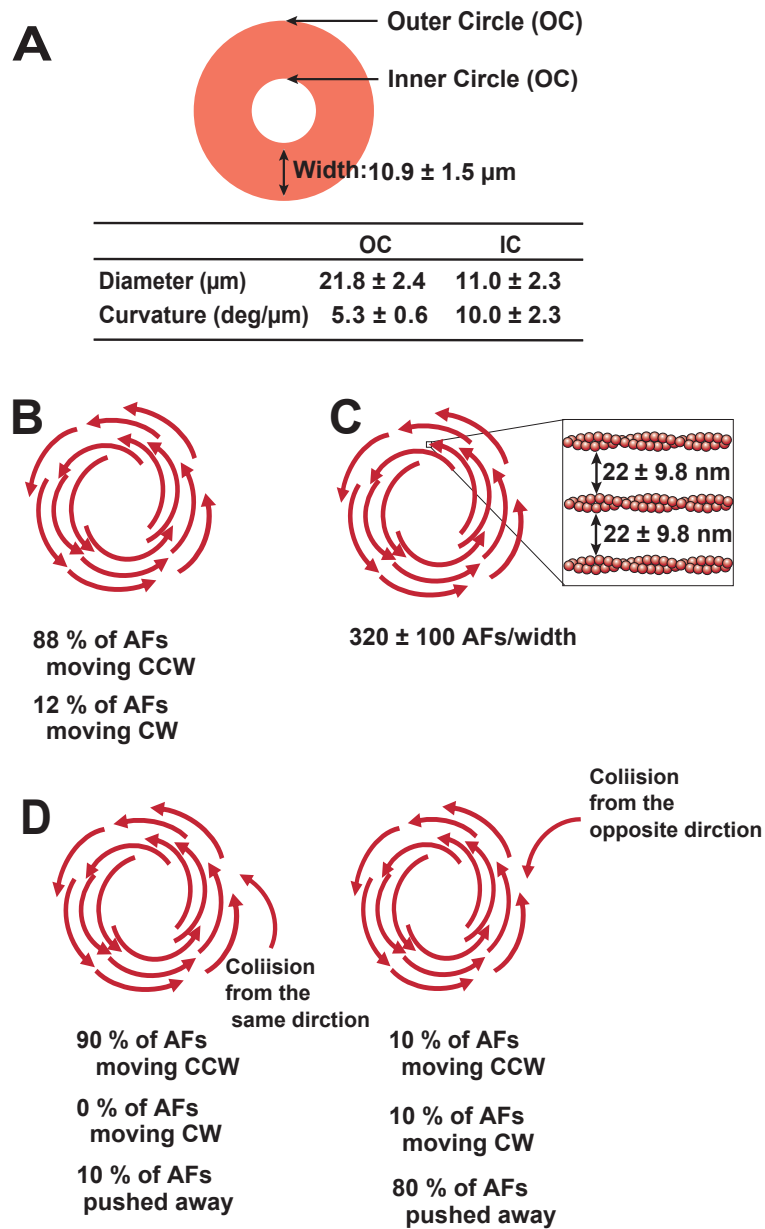
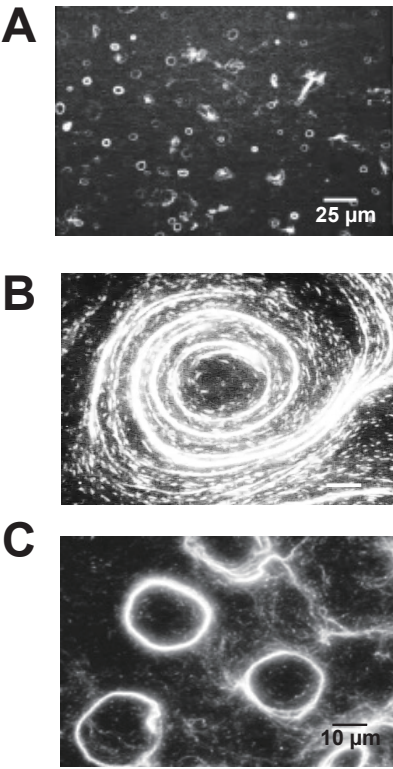


Fig. 3





10 μm