

Cell chirality reversal through tilted balance between polymerization of radial fibers and clockwise-swirling of transverse arcs

Reviewed Preprint

v2 • August 22, 2024

Revised by authors

Reviewed Preprint

v1 • November 29, 2023

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Abstract

Cell chirality is an intrinsic property shown as biased cell rotation or orientation. Although the right-handed double helix of actin is known important, how a single form of molecular handedness manifests diverse forms of cell chirality remains unclear. Here, we found that the cell nucleus rotated with a clockwise (CW) bias in a small projected area, but this rotation reversed to an anticlockwise (ACW) bias as cell spreading increased. Actin analysis suggested that radial fiber polymerization accounts for the ACW bias. Alterations in transverse arc components (myosin II, mDia2, and tropomyosin 4) revealed that the CW bias is driven by the retrograde flow, originating from the tethered gliding motion of myosin II in the contractile structure of transverse arcs. Thus, an imbalance between radial fibers and transverse arcs results in cell chirality reversal. The findings elucidate the mechanisms underlying cell chirality reversal, providing a new perspective on mechanobiology.

eLife assessment

The intrinsic chirality of actin filaments (F-actin) is implicated in the chiral arrangement and movement of cellular structures, but it was unknown how opposite chiralities can arise when the chirality of F-actin is invariant. Kwong et al. present evidence that two actin filament-based cytoskeletal structures, transverse actin arcs and radial stress fibers, drive clockwise and anti-clockwise rotation, respectively. This **fundamental** work, which has broad implications for cell biology, is supported by **compelling** data.

<https://doi.org/10.7554/eLife.92632.2.sa2>

Introduction

The development of an animal's tissues or organs necessitates the establishment of left–right (LR) asymmetry (Lobikin et al., 2012 [DOI](#)). This includes the LR body axis that is vital for visceral distribution (Pohl and Bao, 2010 [DOI](#); Wang et al., 2012 [DOI](#)) and chiral morphogenesis, crucial for the formation of tissue/organ architecture (Hozumi et al., 2006 [DOI](#); Noël et al., 2013 [DOI](#); Spéder et al., 2006 [DOI](#); Veerkamp et al., 2013 [DOI](#)). However, the mechanism underlying the spontaneous emergence of LR asymmetry remains largely unknown. Cell chirality, a mechanical behavior shown as biased cell orientation or rotation (either clockwise (CW) or anticlockwise (ACW)), has been proposed as the origin of LR asymmetry at the tissue level (Danilchik et al., 2006 [DOI](#); Lobikin et al., 2012 [DOI](#)). For example, blastomeres in *Caenorhabditis elegans* embryos exhibit a chiral skew event, leading to uneven cell distribution (Naganathan et al., 2014 [DOI](#); Pohl and Bao, 2010 [DOI](#)). In a later stage of embryonic development, this chiral mechanics causes collective motion in epithelial morphogenesis (Hozumi et al., 2006 [DOI](#); Sato et al., 2015 [DOI](#); Taniguchi et al., 2011 [DOI](#)), eventually affecting the formation of genitalia (Sato et al., 2015 [DOI](#)) and the axial torsion of the hindgut (Hozumi et al., 2006 [DOI](#); Taniguchi et al., 2011 [DOI](#)). In addition to its role in embryonic development, the presence of cell chirality can be recognized by its ability to generate single cellular torque (Liu et al., 2016 [DOI](#); Naganathan et al., 2014 [DOI](#)), promote cell migration with LR bias (Chen et al., 2012 [DOI](#); Xu et al., 2007 [DOI](#); Yao and Ding, 2020 [DOI](#); Yao et al., 2021 [DOI](#)) or form specific alignment at the multicellular level (Chen et al., 2012 [DOI](#); Hu et al., 2018 [DOI](#); Wan et al., 2011 [DOI](#)). Through cell–cell communication, the chiral behavior results in the LR-biased assembly of the multicellular structure (Chen et al., 2012 [DOI](#); Chin et al., 2018 [DOI](#)) and regulates the permeability of intercellular junctions (Fan et al., 2018 [DOI](#)).

Actomyosin activity is crucial for chirality at the organ (Spéder et al., 2006 [DOI](#)), tissue (Bertet et al., 2004 [DOI](#)), and cellular levels (Tamada and Igarashi, 2017 [DOI](#)). In *in vivo* systems, the expression of cell chirality can be suppressed by inhibiting actomyosin activity (Noël et al., 2013 [DOI](#)). In *in vitro* systems, the expression of cell chirality depends on actomyosin activity along micropattern boundaries (Chen et al., 2012 [DOI](#); Tee et al., 2015 [DOI](#)). Two classes of actin filaments play major roles in actomyosin activity: dorsal stress fibers and transverse arcs (Hotulainen and Lappalainen, 2006 [DOI](#); Tojkander et al., 2012 [DOI](#)). Dorsal stress fibers, also referred to as radial fibers, have one end anchored to the substrate through a focal adhesion, whereas the other end points radially toward the nucleus. Transverse arcs are curved actin filaments produced by the endwise annealing of myosin bundles, appearing as rings or nearly closed arcs centered around the nucleus. When cultured on micropatterned circles, radial fibers are unidirectionally tilted, forming a distinct chiral pattern of actin filaments (Tee et al., 2015). This pattern drives the chiral torque and rotation of cells (Bao et al., 2020 [DOI](#); Kwong et al., 2019 [DOI](#); Liu et al., 2016 [DOI](#)).

The molecular handedness of actomyosin filaments is essential for cell chirality. For example, formin-dependent actin polymerization has been proposed to cause the righthanded axial spinning of radial fibers, resulting in the ACW chiral pattern of actin filaments (Tamada and Igarashi, 2017 [DOI](#); Tee et al., 2023 [DOI](#); Tee et al., 2015 [DOI](#)). In addition, the overexpression of α -actinin-1 that crosslinks with the actin filament or modulates actin polymerization can lead to CW chirality (Chin et al., 2018 [DOI](#); Djinoovic-Carugo et al., 1999 [DOI](#); Jalal et al., 2019 [DOI](#); Tee et al., 2023 [DOI](#)). However, because an actin filament is formed only as a right-handed double helix, it remains unclear how a single form of molecular handedness can manifest diverse chirality across different cell types (Liu et al., 2016 [DOI](#); Schonegg et al., 2014 [DOI](#); Wan et al., 2011 [DOI](#); Zaatari et al., 2021 [DOI](#)). The role of crosslinking or altered polymerization of right-handed actin filaments in chirality reversal remains vague and unresolved.

In this study, we report the reversal of cell chirality achieved by switching dominance between the two classes of actin fibers. During cell attachment and spreading, we first observed the reversal of chiral rotation of the nucleus, shifting from a CW-biased to an ACW-biased direction. Using micropatterning, we determined that the reversal of chirality was dependent on an increase in the projected cell area. This phenomenon was consistently observed across various cell types. Based on the analysis of actin distribution by using small-molecule drugs, we found that the polymerization of radial fibers originating from their barbed end near the cell edge is essential for ACW chirality. However, when cells were cultured on small islands or an inhibitor was used to suppress radial fibers, transverse arcs displayed a well-organized CW swirl approaching the cell center. Furthermore, perturbations in transverse arcs, either through the suppression of myosin IIa activity or gene silencing of mDia2 and tropomyosin 4 (Tpm4), enhanced ACW chirality. Because transverse arcs are formed at the dorsal surface of the cell cortex, the sarcomere-like contractile structure may serve as a torque dipole that rolls against the plasma membrane, eventually leading to the CW swirling of transverse arcs. Thus, an imbalance between the two classes of actin stress fibers results in the reversal of chirality. This study elucidated a general and previously unidentified mechanism underlying chirality reversal, providing a unique opportunity to further explore the role of cell chirality in regulating tissue morphogenesis.

Results

Cell chirality is reversed from CW to ACW bias during cell spreading

Attachment to a cell-adherent substrate is a prerequisite for expressing cell chirality in 2D culture (Chen et al., 2012 [DOI](#); Wan et al., 2011 [DOI](#)). We performed time-lapse microscopy to record cell spreading and nuclei rotation during the spreading of human foreskin fibroblasts (HFF-1) on a culture dish. From 0 min to 240 min, cells underwent isotropic spreading and then anisotropic spreading until reaching their normal morphology on the culture dish (**Figure 1A** [DOI](#); **Video 1** [DOI](#)). To assess the cell chirality, cell nuclei were tracked using binarized nuclei images to record the angular velocity of nuclei at 5 min intervals (**Figure 1C and D** [DOI](#)). Notably, the nucleus rotation transitions several times from CW to ACW (**Figure 1D** [DOI](#)). To determine potential chiral bias, we measured the angular velocity of 26 cells and calculated the percentage of CW and ACW rotation every 20 minutes throughout the timelapse imaging. Our results revealed that nucleus rotation was CW-biased at the beginning but reversed to ACW-biased over time (**Figure 1E** [DOI](#)). Additionally, the cell projected area increased (**Figure 1F** [DOI](#)) and the circularity of cell morphology decreased (**Figure 1G and H** [DOI](#)). Because cells with non-circular shape would inhibit the expression of chiral rotation of cells (Liu et al., 2016 [DOI](#)), the increased projection area is more likely the reason for chirality reversal. To clarify this, the probability of ACW/CW rotation was further analyzed against the averaged projected area with different circularity. For circularity greater than 0.7, reversal of chirality was observed (**Figure 1I** [DOI](#)). In contrast, reversal was not as apparent for cell circularity below 0.7 (**Figure 1J** [DOI](#)). Together, these results suggest that chiral nucleus rotation may require a circular cell shape and can be reversed from CW to ACW with the increase of cell projected area.

Cell chirality is reversed from CW to ACW bias with incremental change of micro-island area

During cell attachment and spreading, the actin cytoskeleton undergoes a complete reorganization, such as formation of focal adhesions, recruitment of formin, maturation of actin filament stress fibers, and activation of Arp2/3 in lamellipodia. Thus, the chirality reversal may be a result of those dynamic processes but not necessarily the cell projected area in the transient period. To directly assess the role of the cell projected area, we confined the cell spreading in an isotropic form through applying arrays of fibronectin-coated circular islands with different areas, surrounded by cell-repellent Pluronic coating (**Figure 2A** [DOI](#)). On fibronectin-coated micro-islands,

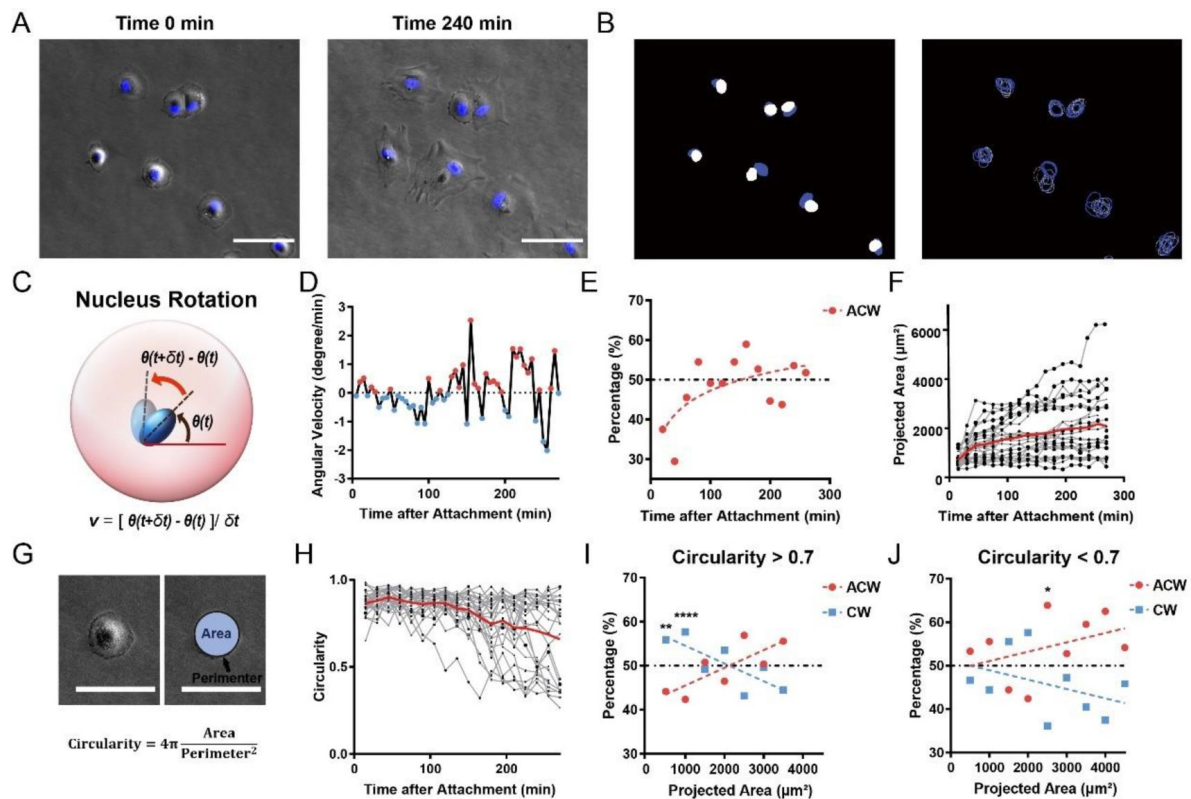


Figure 1.

Rotational bias of cell nuclei reversed from CW to ACW bias during cell attachment.

(A) Phase contrast and H33342 staining of nuclei (blue) at 0 min and 240 min of the time-lapse imaging (Video 1). (B) Imaging processing of nuclei tracking. (Left) After binarization of nuclei, the ellipses of nuclei were shown for time point 0 min (white) and 240 min (blue). (Right) The boundary of cell nuclei at different time points, demonstrating the tracking of nuclei from the beginning to the end point, colored with a gradient from white to blue. (C) Schematic showing calculation of angular velocity using the angle difference of the nucleus at two consecutive images, 5 min per interval for 4 hour and 30 min. (D) The angular velocity of the nucleus measured for one cell. (E) Probability of ACW rotation of nuclei for each 20 min interval ($n = 26$). (F) Cell projected areas of each cell measured by phase contrast images after initial attachment of cells, with 20 min interval (mean shown in red). (G) Circularity calculated by the area and perimeter of cells. (H) Circularity of each cell calculated with 15 min interval (mean shown in red). (I and J) Probability of ACW/CW rotation of nuclei against cell projected area with circularity greater than 0.7 (I) or less than 0.7 (J). Two-sample equal variance two-tailed t-test. Scale bar: 100 μm .

time-lapse measurement started after the cells had fully spread to the defined area without further morphological dynamics. HFF-1 fibroblasts were again used to record nucleus rotation on micro-islands. Interestingly, when cells spread on the $500\ \mu\text{m}^2$ pattern, the nucleus rotation was neutral (**Figure 2B**). Starting from $750\ \mu\text{m}^2$, nucleus rotation became CW-biased ($45.83 \pm 1.96\%$ ACW with $p\text{-value} < 0.01$), which was consistent with the chiral bias of cells during spreading on culture dish. From $1000\ \mu\text{m}^2$ to $2500\ \mu\text{m}^2$, the chiral nucleus rotation changed to neutral and then reversed to ACW-biased ($63.43 \pm 2.90\%$ ACW with $p\text{-value} < 0.0001$) on $2500\ \mu\text{m}^2$ of micro-islands. Thus, the results on micro-islands provide direct evidence that cell projected area was responsible for chirality reversal. To further prove that high circularity is required for area-dependent chirality reversal, cells were cultured on other non-circular shapes, including a triangle, square, and rectangle, with areas exhibiting CW ($750\ \mu\text{m}^2$) or ACW ($2500\ \mu\text{m}^2$) bias on circular islands. The reversal of cell chirality from small to large island was not observed in these cases, indicating that circular islands are required (**Figure 2—figure supplement 1**). Furthermore, to test the universality of such phenomenon, we performed nucleus rotation analysis on two other cell types (**Figure 2C to D**), C2C12 mouse myoblasts and human mesenchymal stem cells (hMSCs). All assessments showed the same chirality reversal depending on cell projected area, demonstrating the universality of this relationship.

Actin distribution upon drug treatment

To investigate the mechanism, we next examined the actin cytoskeleton on the aforementioned islands. Actomyosin cytoskeleton is known to play important role in the cell chirality (**Chen et al., 2012**; **Wan et al., 2011**). More importantly, when cultured as single cells on micropatterned circles, a chiral swirling pattern of radial actin fibers has also been reported (**Tee et al., 2015**). We first observed the actin structure of cell spreading on circular pattern that exhibiting ACW ($2500\ \mu\text{m}^2$, large pattern) and CW ($750\ \mu\text{m}^2$, small pattern) bias. When cultured on the $2500\ \mu\text{m}^2$ pattern (**Figure 3A**, left), actin structure at the lamella arises as a chiral swirling pattern of radial fibers in conjunction with the transverse arcs (**Hotulainen and Lappalainen, 2006**; **Tojkander et al., 2012**). Using automated image processing to analyze the tilting angle of radial fibers with respect to the radial axis of each cell (**Bao et al., 2020**), an ACW tilting was revealed (**Figure 3—figure supplement 1**), suggesting the role of actin in nucleus rotation. To clarify the causal relationship between the nucleus and the actin structure, we observed actin organization of cell seeded on micropatterns after enucleation. By comparing actin filaments for the normal and enucleated HFF-1 fibroblasts on $2500\ \mu\text{m}^2$ island, the ACW bias of actin filament was unchanged (**Figure 3—figure supplement 2**). These results suggest that the chiral rotation of the nucleus is likely a result of chiral structure of actin, rather than the chiral rotation of the nucleus causing the formation of actin chiral structure.

On the other hand, for cells on the $750\ \mu\text{m}^2$ pattern, CW rotation of nucleus was observed (**Figure 2B**). However, only ventral fibers and transverse arcs remained in these cells, while radial fibers were significantly reduced (**Figure 3B**, left). To visualize this structural difference, we used image stacking (see fluorescent staining in the method). On $2500\ \mu\text{m}^2$ pattern, the highest intensity showed a ring shape where the radial fibers were located (**Figure 3A**, right). In contrast, on $750\ \mu\text{m}^2$ pattern, the actin ring was replaced by an accumulation of actin at the cell center (**Figure 3B**, right).

To further analyze the actin structure, we calculated the structures' pixel number and the percentage of radial fibers and transverse arcs. The pixel number of radial fibers is greatly reduced by 91.98 % on $750\ \mu\text{m}^2$ compared to that on the $2500\ \mu\text{m}^2$ pattern, while the pixel number of transverse arc is reduced by 70.58 % (**Figure 3- figure supplement 3A**). Additionally, we compared the percentage of actin structures on different pattern sizes (**Figure 3- figure supplement 3B**). On $2500\ \mu\text{m}^2$ pattern, the percentage of radial fiber in the actin structure is $61.76 \pm 2.77\%$. While on $750\ \mu\text{m}^2$ pattern, it only accounts for $31.13 \pm 2.76\%$. These results provide evidence of the reduction of radial fibers on a smaller pattern.

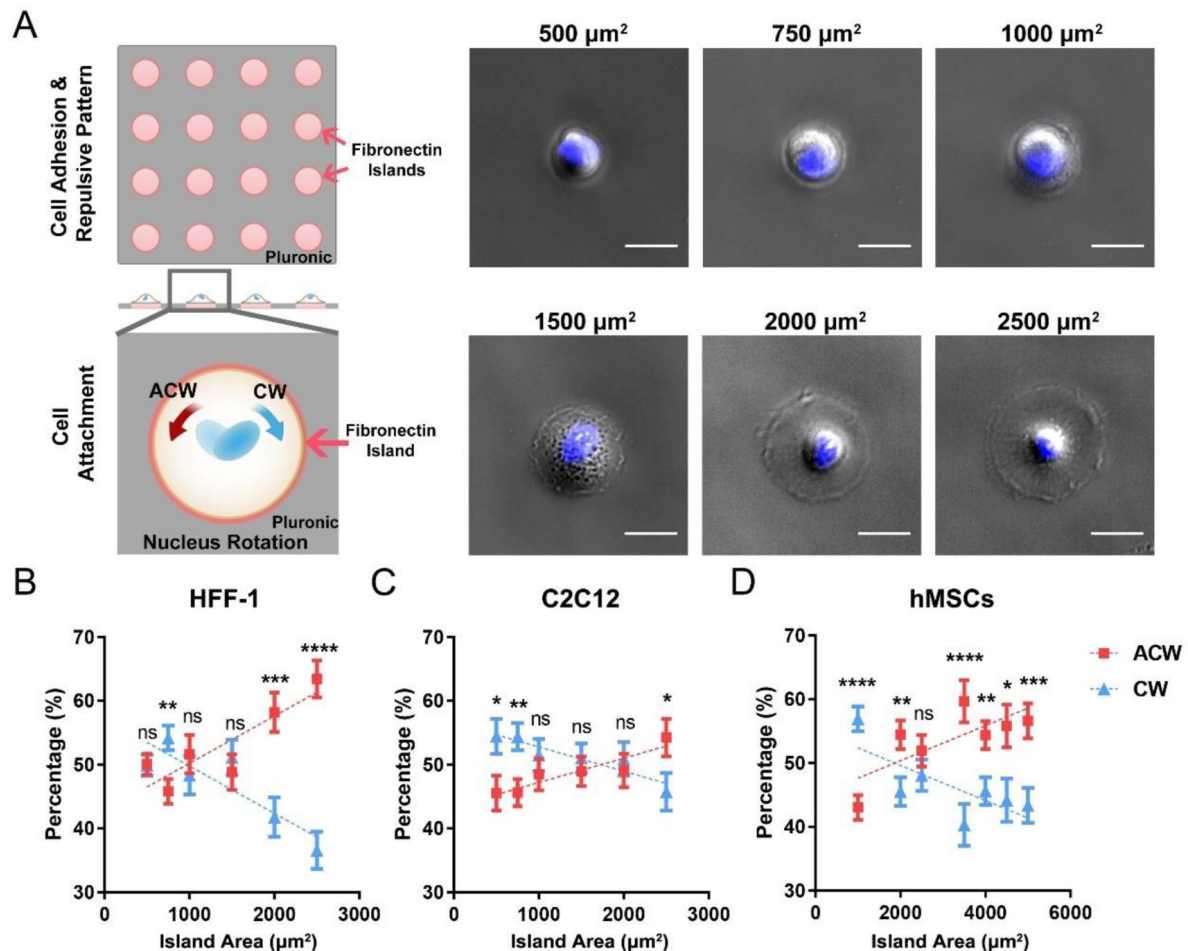


Figure 2.

Reversal from CW to ACW chirality with increasing micro-island area.

(A) Micropatterned islands of cell-adherent fibronectin and cell-repellent Pluronic F127, with areas from 500 μm^2 to 2500 μm^2 , on which rotational bias was measured based on the angular velocity of the long axis of the binarized nucleus ellipse. After the cell was fully spread on the micropatterned island (2–3 hours), time-lapse images were taken every 10 min for 2–3 hours to measure the angular velocity. (B to D) Probability of ACW/CW rotation of HFF-1 human fibroblasts (B), C2C12 mouse myoblasts (C), and human mesenchymal stem cells (hMSCs) (D) on micro-islands with varied area within 3 hours. Mean $\pm$ SEM. Two-sample equal variance two-tailed t-test.

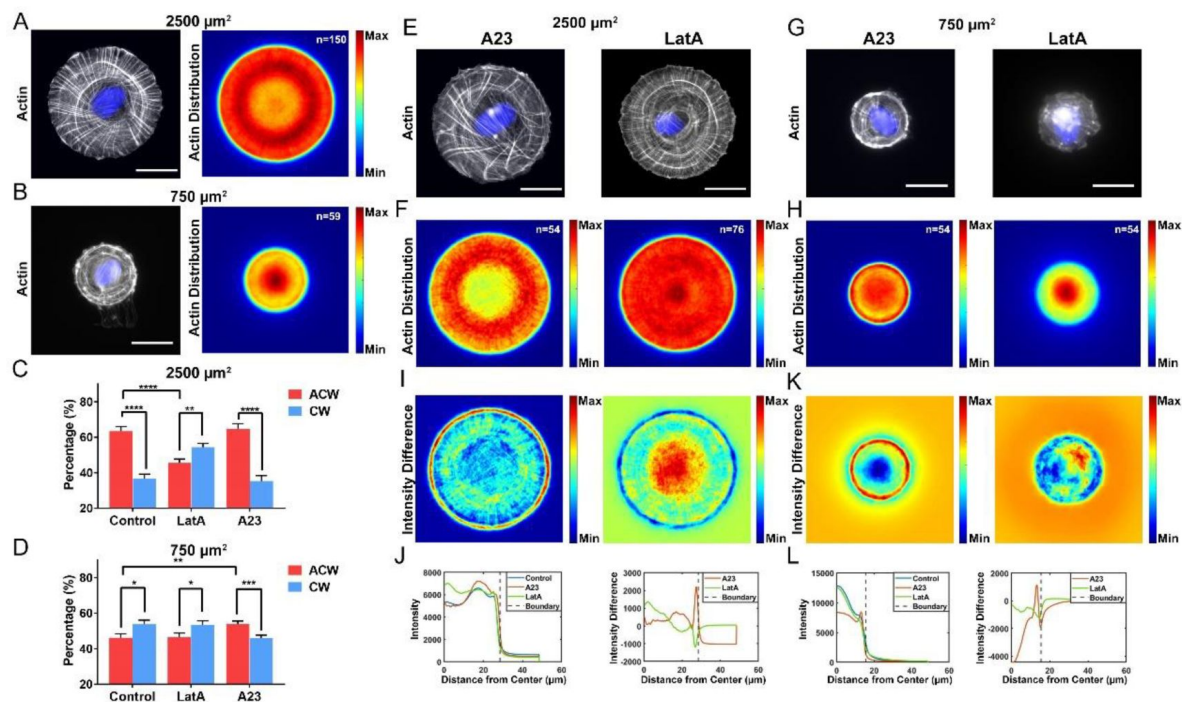


Figure 3.

Actin at cell periphery promotes ACW chirality.

(A) Fluorescent staining of F-actin (white) by phalloidin, DAPI-stained nucleus (blue), and a stacked image showing heatmap distribution on a $2500 \mu\text{m}^2$ island. (B) Fluorescent staining of F-actin (white) by phalloidin, DAPI-stained nucleus (blue), and a stacked image showing heatmap distribution on a $750 \mu\text{m}^2$ island. (C and D) Probability of ACW/CW rotation on (C) $2500 \mu\text{m}^2$ of control ($n = 57$), treatment of A23 ($n = 50$) and LatA ($n = 54$), and (D) $750 \mu\text{m}^2$ for control ($n = 34$) and treatment of A23 ($n = 70$) and LatA ($n = 43$). Mean + SEM. Two-sample equal variance two-tailed t-test. (E) Fluorescent staining of F-actin (white) by phalloidin and DAPI-stained nucleus (blue) with treatment of A23 (left) and LatA (right) on a $2500 \mu\text{m}^2$ island. (F) Stacked image of (E) showing distribution of actin by heatmaps. (G) Fluorescent staining of F-actin (white) by phalloidin and DAPI-stained nucleus (blue) with treatment of A23 (left) and LatA (right) on a $750 \mu\text{m}^2$ island. (H) Stacked image of (G) showing distribution of actin by heatmaps. (I) Differential heatmap showing intensity differences of actin, comparing the treatment of A23 (left) and LatA (right) to the control on a $2500 \mu\text{m}^2$ island. (J) Curves of actin intensity from cell center to boundary (left) and differential actin intensity (right) comparing A23 and LatA treatment to the control on a $2500 \mu\text{m}^2$ pattern. (K) Differential heatmap showing intensity differences of actin, comparing the treatment of A23 (left) and LatA (right) to control on a $750 \mu\text{m}^2$ island. (L) Curves of actin intensity from cell center to boundary (left) and differential actin intensity (right) comparing A23 and LatA treatment to the control on a $750 \mu\text{m}^2$ pattern. Scale bar: $20 \mu\text{m}$.

The greatly reduction of radial fibers is unexpected, as the chiral pattern of radial fibers is a hallmark of cell chirality (Tee et al., 2015). To further investigate this, we applied a series of cytoskeleton-related inhibitors on HFF-1 fibroblasts on two representative micro-islands, 750 μm^2 (small island) and 2500 μm^2 (large island). We identified A23187 Calcium Ionophore (A23) (Shao et al., 2015), Latrunculin A (LatA) (Yarmola et al., 2000), and NSC23677 (NSC) (Levay et al., 2013; Spiering and Hodgson, 2011) to modulate actin cytoskeleton. LatA inhibits F-actin polymerization by forming a 1:1 molar complex with G-actin. Based on previous studies, LatA has demonstrated reversing the chirality at or below 50 nM (Bao et al., 2020; Chin et al., 2018; Kwong et al., 2019; Wan et al., 2011). On the other hand, because inhibiting F-actin assembly can lead to the expression of CW chirality, we hypothesized that the opposite treatment might enhance ACW chirality. Therefore, A23187 was chosen with 2 μM concentration as it could initiate the actin remodeling and stress fiber formation (Shao et al., 2015). Furthermore, in the attempt to replicate the reversal of chirality by inhibiting F-actin assembly through other pathways, we explored NSC23677 at 100 μM , which was found to inhibit the Rac1 activation (Chen et al., 2011; Gao et al., 2004) and reduce cortical F-actin assembly (Head et al., 2003). On 2500 μm^2 island, the original nucleus ACW chirality (63.43 ± 2.90 % ACW) remained unchanged by A23 treatment (Figure 3C) and became even more ACW-biased under NSC (Figure 3—figure supplement 4). In contrast, treatment of LatA could reverse the chirality to CW bias (45.58 ± 2.15 % ACW, Figure 3C). Additionally, CW bias on 750 μm^2 island was unaffected by the LatA treatment (Figure 3D), but neutralized by NSC (Figure 3—figure supplement 4). More interestingly, A23 reversed the nucleus rotation from its original 45.83 ± 1.96 % ACW to 54.00 ± 1.60 % ACW bias (Figure 3D). Based on the above results, treatment of LatA and A23 appears to be effective in reversing the induced ACW/CW bias on large and small island, respectively.

We next analyzed the actin distribution of HFF-1 fibroblasts in response to A23 and LatA treatment. Compared to untreated cells on 2500 μm^2 pattern, actin was lessened around the nucleus with A23 treatment. More ventral stress fibers across the cell nucleus could be seen with LatA treatment (Figure 3E). To statistically illustrate actin distribution upon treatment, we applied image stacking. On 2500 μm^2 pattern, A23 treatment pushes the actin toward the cell edge, while in contrast, the LatA treatment brought actin closer to the center (Figure 3F). Similar effects were seen for cells on 750 μm^2 pattern. More interestingly, radial fibers were restored under A23 treatment (Figure 3G and H). To highlight this difference, we used a differential heatmap by subtracting the stacked actin image between treated and untreated cases. A23 clearly appeared to concentrate actin at the cell edge but reduce it at the center (Figure 3I), and a distinct peak of actin intensity was observed immediately adjacent to the cell edge (dashed line, Figure 3J). In contrast, under LatA treatment, actin was concentrated around the nucleus while the peripheral ring of actin was more reduced than that of the untreated control (Figure 3I and J). The same effects were also observed in cells on 750 μm^2 islands (Figure 3K and L).

As polymerization of radial fibers arose from the barbed end near the leading edge, this suggests an increased polymerization of radial fibers upon A23 treatment, which may account for the ACW chiral rotation. Particularly for cells on 750 μm^2 pattern, it was possible to change the original CW rotation to ACW rotation upon restoration of radial fibers. On the other hand, as an inhibitor of F-actin polymerization, LatA, appeared to reduce actin filament at the cell edge (Higashida et al., 2008) on both 2500 μm^2 and 750 μm^2 patterns, suggesting a suppressed actin polymerization. To validate the altered proportion of actin fibers, we calculated the percentage of radial fibers and transverse arcs with control and treated cells, i.e. A23 treatment on 750 μm^2 islands or LatA treatment on 2500 μm^2 islands. Compared to that of the control, the percentage of radial fibers pixel indeed increased with A23 treatment but decreased with LatA treatment, suggesting the role of polymerization of radial fibers which may account for the ACW chirality (Figure 3—figure supplement 5 and Figure 3—figure supplement 6).

Role of actin polymerization of radial fibers for ACW chirality

How does actin polymerization near the cell edge affect cell chirality? In 2015, Tee, Bershadsky and colleagues introduced the stair-stepping theory, which suggested how radial fiber polymerization generates ACW force and drives the actin cytoskeleton into the ACW pattern (Tee et al., 2015). To better elucidate this mechanism, we visualized live actin organization by transfection of either actin-GFP, or Lifeact, in HFF-1 fibroblasts. Cells on the 2500 μm^2 island were chosen due to their easier observation. We first examined the actin organization of the untreated control cells with Lifeact (Figure 4A). Time-lapse microscopy showed that the centripetal growing radial fiber was pointed toward the cell center with an inward flow of transverse arcs (Figure 4A; Video 2). Notably, in untreated control cells, the radial fibers tilted more rightward with respect to the direction of elongation. Eventually, this right-tilted growth direction of radial fibers, accompanied by retrograde flow, drove an overall ACW rotation of the entire cytoskeleton around the cell nucleus. This resulted in a chiral swirling pattern of actin filament, showing a strong correlation with ACW nucleus rotation.

To better elucidate the role of actin polymerization of radial fibers, we compared the elongation rate of the radial fibers and the retrograde flow velocity of the transverse arcs. Actin-GFP transfected HFF-1 cells on 2500 μm^2 pattern. We observed that the retrograde flow of transverse arcs was faster than the elongation rate of radial fiber (Figure 4B), which is consistent with the literature (Tee et al., 2015). This suggests that the retrograde flow of transverse arcs is not dependent solely on the elongation of radial fibers. More importantly, the velocity of radial fiber elongation was reduced by LatA treatment, while the inward flow velocity of transverse arcs remained unchanged compared to the control (Figure 4C and D). Thus, these results suggest that actin polymerization directly contributes to the elongation of radial fibers but not the retrograde flow of transverse arcs.

Altogether, our results suggest that inward-growing radial fibers with rightward tilt are the main contributors to ACW chirality. When treated by LatA (Figure 4D) or cultured on small islands that suppress the radial fibers (Figure 3B; Video 3), the ACW bias was reversed to CW bias. Notably, rightward tilting during actin polymerization could be explained by the helical structures of actin (Tamada and Igarashi, 2017; Tee et al., 2015). Specifically, in the ‘stair-stepping’ mode proposed by Tee et al., formin proteins cap and tether the barbed ends of actin filaments at focal adhesion. When new actin monomers are incorporated into the right-handed double helix, which drives the other free end with a right-handed axial spinning (Video 4) (Tee et al., 2015).

Transverse arcs contribute to CW chirality

While actin polymerization of radial fibers directly contributes to the ACW rotation of cells, the appearance of CW bias is unclear, as the reduced actin polymerization may simply neutralize chiral bias. For LatA-treated cells with Lifeact transfection, we noticed a very organized CW swirling of transverse arcs that approached the cell center during retrograde flow (Video 5). Using optical flow analysis, such inward flow with CW-bias swirling was observed to drive the cell to commit CW rotation (Figure 4E). This finding explains the increased actin concentration near the cell center shown by phalloidin staining of LatA-treated cells on the large island (Figure 3I). Notably, in some cases, the CW-bias of nucleus rotation occurred only in the presence of transverse arcs that swirled and wrapped towards the cell center with CW-bias, without distinct, bundled radial fibers (Video 6). Moreover, for cells on small islands, the nucleus rotation was solely driven by the retrograde flow of transverse arcs, without apparent radial fibers (Video 3). Thus, such CW swirling of transverse arcs may occur on itself without the need for radial fibers.

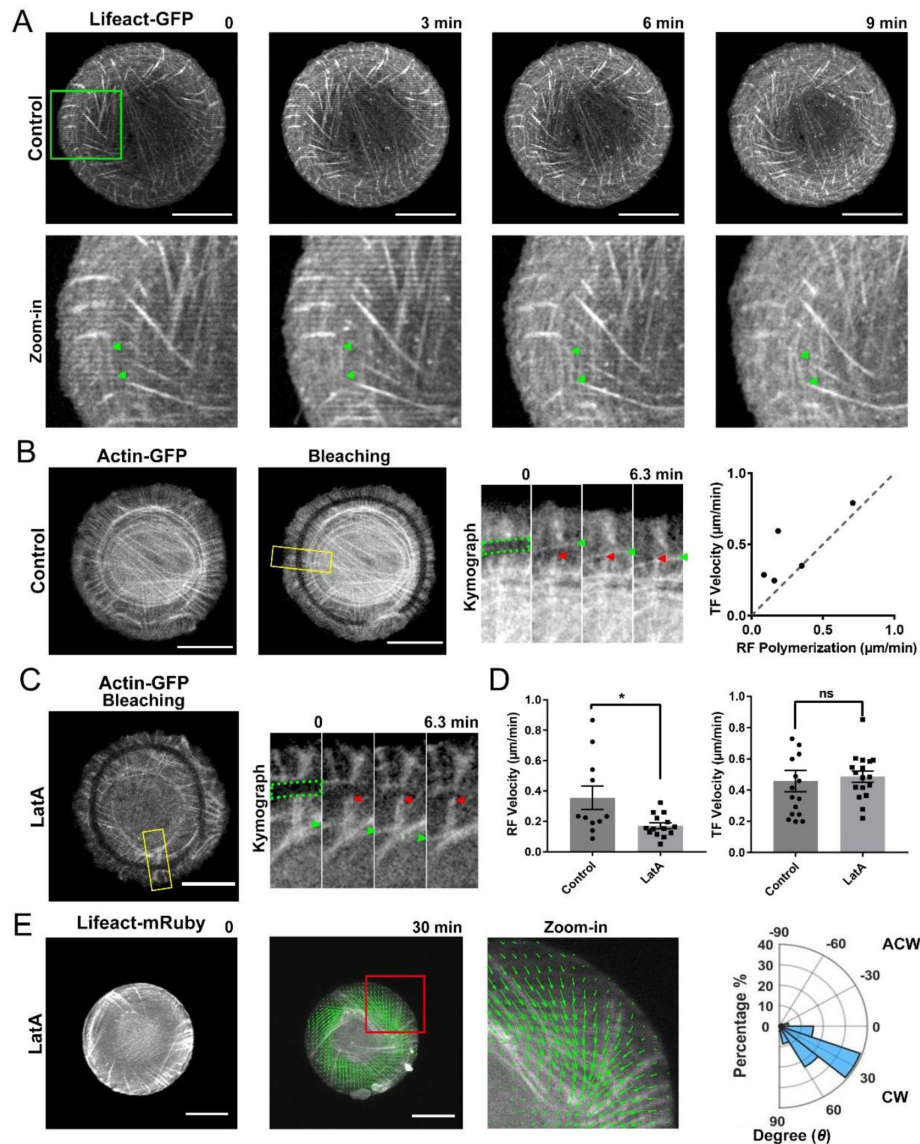


Figure 4.

Polymerization of radial fibers drives ACW chirality.

(A) Lifeact-GFP transfected cell showing actin organization in the control on $2500 \mu\text{m}^2$ circular islands. With the increase in length, the radial fibers start to tilt rightward. Following the established direction, the transverse arcs (green arrows) flow toward the cell center and cause an overall ACW rotation of the entire cytoskeleton. (B and C) Photobleaching in a ring shape parallels the circumference of actin-GFP transfected cells of the untreated control (B) or LatA-treated samples (C). A kymograph (yellow box) of the photobleached untreated control was used to measure the centripetal velocity of transverse arcs and elongation rate of radial fibers. The green dashed line indicates the photobleached location of actin-GFP, while red and green arrows show the elongation of radial fiber and translocation of transverse arc to the cell center, respectively. In each cell sample, radial fiber elongation velocity was compared against the transverse arc translocation velocity. The velocity was taken as the average of 2–3 measurements from different locations of the same cell. (D) The radial fiber elongation velocity (left) and the transverse arc translocation velocity (right) in untreated control and cells with LatA treatment. For radial fiber elongation velocity measurement, $n = 11$ for the control and $n = 14$ for LatA treatment, based on 4–5 cells. For the transverse arc translocation velocity measurement, $n = 16$ for the control and $n = 17$ for LatA treatment based on 4–5 cells. Mean $\pm$ SEM. Two-sample equal variance two-tailed t-test. (E) Lifeact-mRuby transfected cell showing actin organization of $2500 \mu\text{m}^2$ circular pattern with LatA treatment. Motion vectors (green) demonstrate the accumulated motion of the actin structure. Polar histogram shows the distribution angle of motion vectors, indicating the transverse arcs' CW swirling. Scale Bar: $20 \mu\text{m}$.

To further investigate this, we disturbed the major components of the transverse arcs. Transverse arcs are comprised of curved actomyosin bundles (Tamada and Igarashi, 2017; Tee et al., 2015) form in a periodic pattern through end-to-end annealing of myosin II and α -actinin (Tojkander et al., 2012). Importantly, myosin II bundles contribute to transverse arc contraction by walking on actin filament, which may lead to the higher speed in retrograde flow of transverse arcs (Figure 4B). Thus, myosin II or α -actinin perturbation would directly influence the transverse arc.

We first applied overexpression of α -actinin. We found that the overexpression of α -actinin enhanced CW rotation (Figure 5—figure supplement 1). Such enhancement the CW chirality was theorized by ‘screw-step’ motion (Tee et al., 2015) because it cross-links radial fibers, which may restrict and suppress their axial spinning in ACW chirality. Alternatively, as the main component of transverse arcs, α -actinin may simply enhance its formation, leading to enhanced CW chirality.

To further validate the role of transverse arcs in CW chirality, we applied Y27632, an inhibitor of Rho-associated kinase (ROCK), to down-regulate the function of myosin II (Salhia et al., 2008). Interestingly, treatment with Y27632 altered the actin arrangement and significantly reduced the highly enriched myosin II in transverse arcs observed in untreated cells, showing that actomyosin activity was disrupted (Figure 5A). We then compared actin and myosin II intensity distribution using a heatmap of a $2500\ \mu\text{m}^2$ pattern. For non-treatment control (Figure 5B), myosin II was distributed with the highest intensity as a ring and colocalized with the actin ring. Remarkably, in the Y27632 treated cell (Figure 5B), although the actin arrangement was altered, the actin ring persisted. However, the myosin ring was heavily damaged and failed to colocalize with the actin ring. To quantify this, we analyzed myosin II median intensity and its colocalization with actin filament using Pearson’s R-value (Figure 5C and D), in which a higher value of median intensity indicated more activated myosin II, and higher R-value indicated more myosin II colocalized with actin filaments. Significant reductions in myosin II intensity and its colocalization with actin filament were seen upon Y27632 treatment. To illustrate the myosin II distribution, the myosin II intensity profiles were plotted to compare the control and Y27 treatment, further supporting the disruption of myosin ring (Figure 5—figure supplement 2).

With such disruption of myosin II function, we measured nucleus rotation under Y27632 treatment on islands $2500\ \mu\text{m}^2$ and $750\ \mu\text{m}^2$. The ACW nucleus rotation was enhanced on both islands (Figure 5E and F). Similarly, this enhanced ACW rotation observed in cells treated with NSC (Figure 3—figure supplement 4) can also be explained by the cross-inhibition to myosin II (Shibata et al., 2015). In summary, these results suggest that myosin II activity of transverse arc is responsible for the CW-bias of cells.

Role of myosin IIa and transverse arc on CW chirality

To further investigate the role of myosin IIa and transverse arc in the CW chirality, we applied siRNA gene silencing on mDia2. mDia2 typically nucleates tropomyosin-decorated actin filaments, which recruit myosin II and anneal endwise with α -actinin-crosslinked actin filaments (Tojkander et al., 2011). The sarcomere-like contractile unit eventually condenses to myosin II-containing transverse arcs (Tojkander et al., 2011). We first compared the myosin IIa distribution of the control (Figure 6A) and mDia2-silenced cells. We reduced the protein expression upon siRNA (Figure 6B). Interestingly, while the myosin ring was heavily disrupted by mDia2 silencing, some myosin IIa was still observed at the region of transverse arcs (Figure 6C). In addition, more actin is distributed at the cell periphery, but the actin ring remains unchanged. Myosin IIa median intensity analysis and its colocalization with actin show that myosin IIa only partially dislocates from the actin ring, without significant reduction of its intensity (Figure 6D). Regarding nucleus rotation, mDia2 silencing appears to promote ACW rotation on both $2500\ \mu\text{m}^2$ and $750\ \mu\text{m}^2$ islands (Figure 6E and F). On the other hand, an opposite effect was seen by

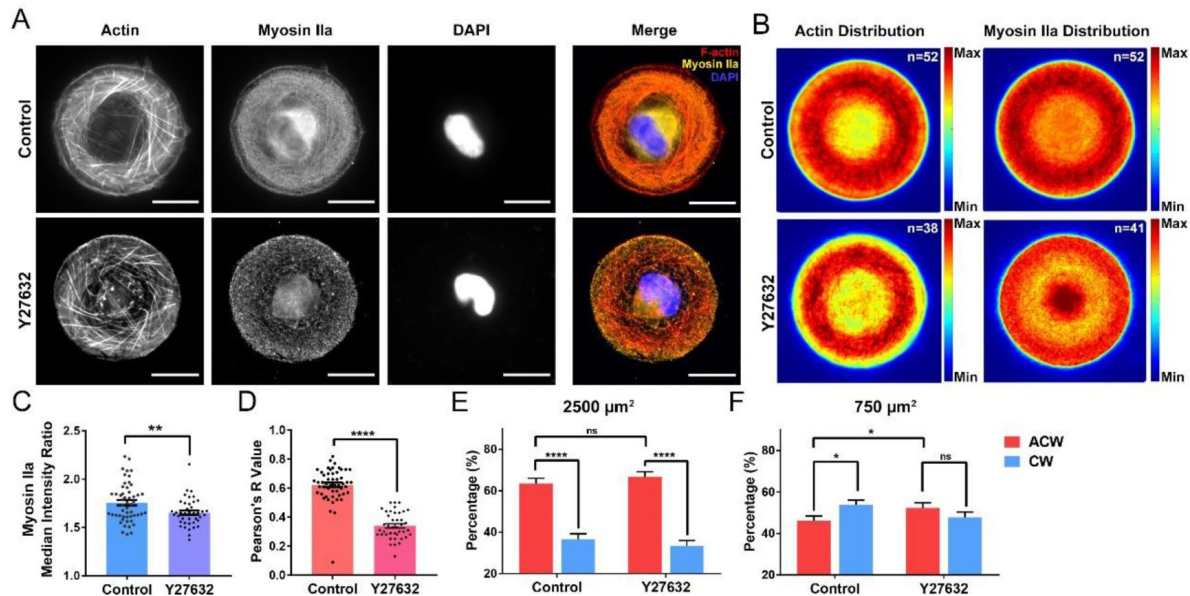


Figure 5.

Inhibition of myosin II enhances ACW chirality.

(A) Fluorescence staining of F-actin (red) by phalloidin, immunofluorescence-stained myosin IIa (yellow), and DAPI-stained nucleus (blue) of the control and cells with myosin II inhibitor Y27632 a 2500 μm^2 pattern. (B) Stacked image of (A) showing heatmap distribution of actin (left) and myosin IIa (right). (C) The median intensity of myosin IIa in control ($n = 52$) and Y27632-treated ($n = 41$) cells. Mean $\pm$ SEM. Two-sample equal variance two-tailed t-test. (D) Colocalization analysis by Pearson's R value of myosin IIa on actin filaments in control ($n = 52$) and Y27632-treated ($n = 41$) cells. Mean $\pm$ SEM. Two-sample equal variance two-tailed t-test. (E) Probability of ACW/CW rotation of control (reused data from [Figure 3D](#), $n = 57$) and Y27632-treated ($n = 53$) cells on 2500 μm^2 pattern. (F) Probability of ACW/CW rotation of control (reused data from [Figure 3E](#), $n = 34$) and Y27632-treated ($n = 53$) cells on 750 μm^2 pattern. Mean $\pm$ SEM. Two-sample equal variance two-tailed t-test. Scale Bar: 20 μm .

transfection of GFP-mDia2 in the overexpression assay. Thus, myosin IIa binding on tropomyosin decorated actin is required for CW nucleus rotation, and mDia2 can serve as an upstream endogenous factor to regulate it.

To further prove the essence of myosin II binding on the tropomyosin-decorated actin, we applied siRNA gene silencing on tropomyosin 4 (Tpm4). Among the isoforms, Tpm4-decorated actin filaments were particularly important for recruitment of myosin II, as Tpm4's presence on the transverse arcs coincides with the incorporation of myosin II. As an essential facilitator for the gliding motion of myosin II on actin filaments, tropomyosin would shift to cover or disclose the myosin-binding site on the actin filament (Ebashi, 1972) and accelerate myosin head translocation to each of the myosin-binding sites (Hundt et al., 2016). We found that Tpm4 was colocalized with actin filaments (Figure 6—figure supplement 1) and could be significantly reduced upon siRNA treatment (Figure 6B). Interestingly, based on the distribution heatmap of myosin IIa, the ring pattern in control (Figure 6A) was eliminated in Tpm4-silenced cells (Figure 6G). Moreover, the transverse arcs became less apparent, and the actin ring was closer to the periphery of cells (Figure 6G). Notably, myosin IIa intensity and colocalization were both statistically lower in Tpm4-silenced cells (Figure 6H), implying that the Tpm4 silencing strongly disrupts the attachment of myosin II on actin filaments. We compared the myosin IIa distribution by the myosin intensity profiles (Figure 6—figure supplement 2), which showed a greater disruption in Tpm4-silenced cells compared to that in mDia2 silenced cells. Compared to the silencing of upstream mDia2, under which actin condensation still occurs, Tpm4 silencing seems to have more pronounced effect in breaking the binding of myosin IIa on actin filaments. As a result, the condensation of actin filaments in the transverse arcs was also damaged. Critically, the nucleus rotation of HFF-1 under Tpm4 silencing exhibited much greater ACW chirality on both the $2500\ \mu\text{m}^2$ (from $63.43 \pm 2.90\%$ ACW to $81.64 \pm 2.79\%$ ACW, Figure 6I) and $750\ \mu\text{m}^2$ patterns (from $45.83 \pm 1.96\%$ ACW to $61.37 \pm 3.09\%$ ACW, Figure 6J). In contrast, Tpm1, Tpm2, and Tpm3 knockdown did not show any noticeable effect on chirality (Figure 6—figure supplement 3). Based on the above results, we can conclude that the myosin IIa binding and its gliding motion on Tpm4decorated actin filament is important for cell expression of CW chirality.

Actin height profile disclosed the transverse arcs and radial fibers layering

Myosin II and V have been shown to be left-handed spiral motors on the right-handed actin helix (Ali et al., 2002; Beausang et al., 2008). During the gliding motion, the left-handed walking of myosin II (Kovacs et al., 2007) could, on the other hand, cause left-handed twirling of an actin filament about its axis (Beausang et al., 2008). When near a surface or a boundary, such chiral processes cause spinning of individual filopodia of a growth cone (Tamada et al., 2010), chiral flow of the cell cortex (Danilchik et al., 2006; Furthauer et al., 2013; Pohl and Bao, 2010), and right-handed clockwise cell migration (Tamada and Igarashi, 2017). Thus, the contractile unit composed of left-handed-walking myosin II on left-handed-twirling of actin filaments may also serve as active actomyosin torque dipoles. If located near a surface, such as the cell cortex, actomyosin torque dipoles could cause right-screw rotation against the surface, eventually causing the transverse arc to swirl in the CW direction.

To explore the location of the transverse arc, we observed the actin structure and myosin distribution in the height profile. According to previous findings, the actin network has two distinct, vertically separated layers. For example, in U2O2 cells, dorsal stress fibers, also called radial fibers attached to focal adhesion, are in the lower layer, while the contractile actin arcs are in the upper layer (Burnette et al., 2014). With fluorescence staining of actin and immunofluorescence staining of myosin IIa, we observed the actin structure and myosin IIa using z-stack with confocal microscopy. The maximum projection and height profiles were created using ImageJ. We found that the maximum projection of actin and myosin IIa was colocalized (Figure 7A and B). More importantly, based on the height profile from ImageJ, two distinct layers of actin,

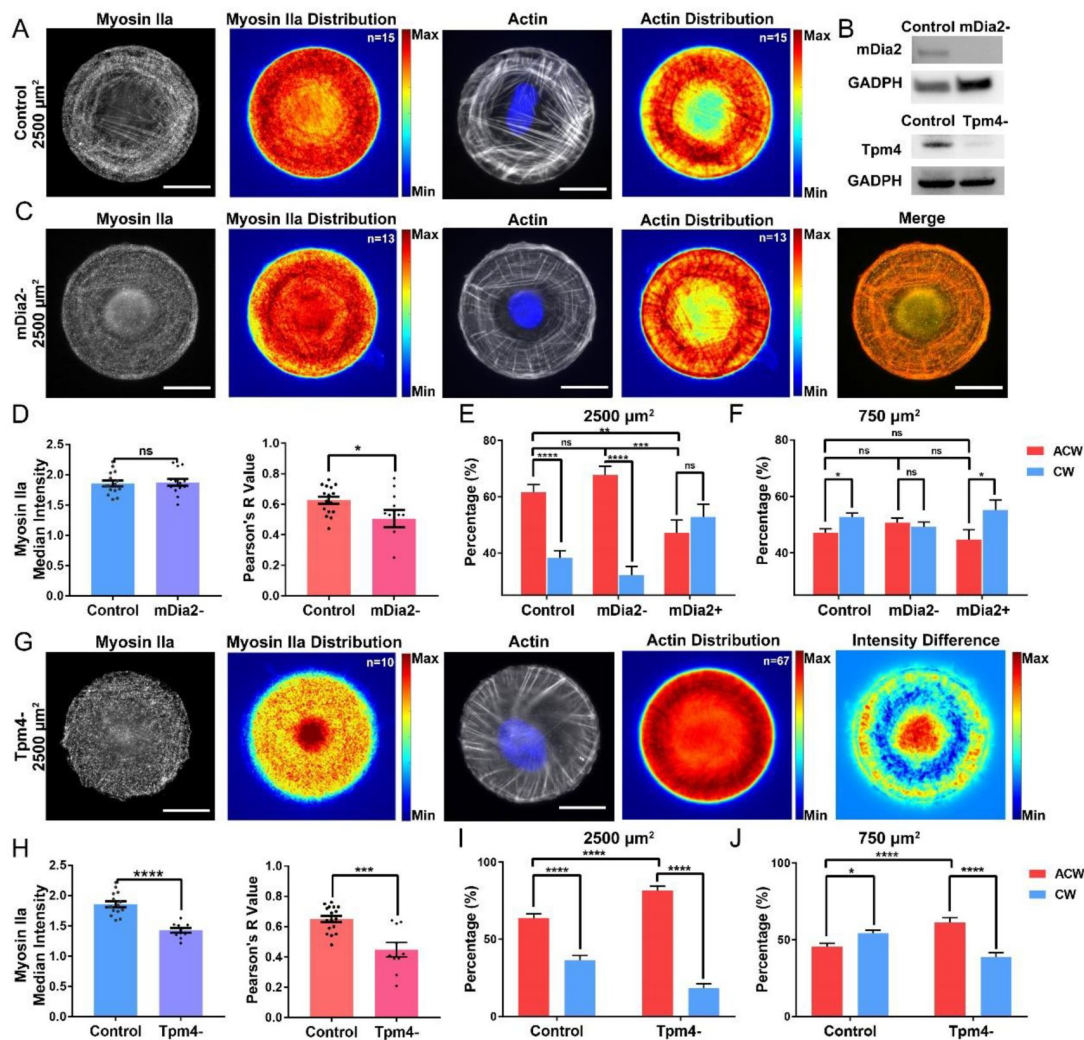


Figure 6.

Myosin IIa binding on actin fibers drives CW chirality.

(A) Fluorescent staining of control on a 2500 μm^2 micropattern with myosin IIa (left) and phalloidin-stained F-actin (right) with DAPI (blue), with the corresponding heatmap. (B) Western blotting validating the silencing effect of mDia2 (top) and Tpm4 (bottom) siRNA transfection compared to the control. (C) Fluorescent staining of mDia2-silenced HFF-1 cell on a 2500 μm^2 micropattern with myosin IIa (yellow) and phalloidin-stained (red) F-actin with the corresponding distribution heatmap. (D) The myosin IIa intensity analysis by the median intensity (left) and colocalization analysis by Pearson's R-value (right), comparing the control ($n = 15$) and mDia2-silenced ($n = 13$) samples regarding the intensity of myosin IIa images and the colocalization of myosin IIa with actin fluorescence images. Mean $\pm$ SEM. Two-sample equal variance two-tailed t-test. (E) Probability of ACW/CW rotation on 2500 μm^2 pattern with untreated control (reused data from Figure 3D, $n = 57$), mDia2 silencing ($n = 48$), and overexpression ($n = 25$). (F) Probability of ACW/CW rotation on 750 μm^2 pattern with untreated control (reused data from Figure 3E, $n = 34$), mDia2 silencing ($n = 53$), and overexpressing ($n = 22$). Mean $\pm$ SEM. Two-sample equal variance two-tailed t-test. (G) Fluorescent staining of Tpm4-silenced HFF-1 cell on a 2500 μm^2 pattern with myosin IIa (yellow) and phalloidin-stained (red) F-actin with DAPI (blue), and the corresponding distribution heatmap. Differential heatmap shows the intensity difference between the actin of the Tpm4-silenced cells and the control on a 2500 μm^2 pattern (right). (H) The myosin IIa intensity analysis by the median intensity (left) and colocalization analysis by Pearson's R-value (right), comparing the control (reused data from (C), $n = 15$) and Tpm4 silenced ($n = 10$) samples in the intensity of myosin IIa images and the colocalization of myosin IIa with actin fluorescence. Mean $\pm$ SEM. Two-sample equal variance two-tailed t-test. (I) Probability of ACW/CW rotation on 2500 μm^2 pattern with untreated control (reused data from Figure 3D, $n = 57$) and Tpm4-silenced cells ($n = 44$). (J) Probability of ACW/CW rotation on 750 μm^2 pattern with untreated control (reused data from Figure 3E, $n = 34$) and Tpm4-silenced cells ($n = 48$). Mean $\pm$ SEM. Two-sample equal variance two-tailed t-test. Scale Bar: 20 μm . GAPDH was used as a loading control in (B).

one near the ventral substrate and one near to the dorsal surface of cells, were seen (green arrows in **Figure 7A and B**). However, myosin IIa was only seen near the dorsal surface, suggesting that the transverse arc was formed at the dorsal layer (**Figure 7A**, right; **Video 7**). We then investigated the actin structure of the Tpm4- silenced sample. A distinct layering of actin could again be observed with z-direction scanning (**Figure 7C**; **Video 8**). To visualize the structure, part of the actin was zoomed in on, and the level of actin was given different colors. It was observed that the radial fibers near the cell periphery were indeed located at the ventral surface, and the transverse arcs, with connection to the extension of radial fibers, were near the dorsal surface of the cells. Together, these results suggest that the CW-bias of transverse arcs could be a result of chiral processes near the dorsal surface. The transverse arcs could be observed above the nucleus with conjunction to a perinuclear actin cap (**Figure 7—figure supplement 1**). Thus, the transverse arcs' CW-bias swirling and wrapping may eventually exert force causing nucleus rotation.

Discussion

In this study, we first observed chiral reversal from CW to ACW bias during cell spreading. Using micropatterning, the CW bias of nucleus rotation on smaller islands was reversed to ACW bias with increased area of micro-islands, suggesting a dependence on cell projected area. We examined nucleus rotation and actin distribution with various actin-related drugs. Examination of radial fibers and their elongation rate indicated that actin polymerization plays a role in ACW chirality. In contrast, CW bias was associated with transverse arcs. Using time-lapse microscopy, we found that the transverse arcs swirl CW during retrograde flow, causing the nucleus to commit to the CW-biased rotation. Further study indicates that the binding of myosin IIa and actin at transverse arcs near the dorsal surface of cells is required for the CW bias.

ACW chirality can be explained by the right-handed axial spinning of radial fibers during polymerization, i.e. 'stair-stepping' mode proposed by Tee *et al.* (Tee *et al.*, 2015) (**Figure 8A**; **Video 4**). As actin filament is formed in a right-handed double helix, it possesses an intrinsic chiral nature. During the polymerization of radial fiber, the barbed end capped by formin at focal adhesion was found to recruit new actin monomers to the filament. The tethering by formin during the recruitment of actin monomers contributes to the right-handed tilting of radial fibers, leading to ACW rotation. Supporting this model, Jalal *et al.* (Jalal *et al.*, 2019) showed that the silencing of mDia1, capZB, and profilin 1 would abolish the ACW chiral expression or reverse the chirality into CW direction. Specifically, the silencing of mDia1, capZB or profilin-1 would attenuate the recruitment of actin monomer into the radial fiber, with mDia1 acting as the nucleator of actin filament (Tsuji *et al.*, 2002), CapZB promoting actin polymerization as capping protein (Mukherjee *et al.*, 2016), and profilin-1 facilitating ATP-bound G-actin to the barbed ends (Haarer and Brown, 1990; Witke, 2004). The silencing resulted in a decrease in the elongation velocity of radial fiber, driving the cell into neutral or CW chirality. These results support our findings that reduction of radial fiber elongation can invert the balance of chirality expression, changing the ACW-expressing cell into a neutral or CW-expressing cell.

On the other hand, our results further suggest that the chiral structure of the transverse arc could be responsible for CW-swirling. The actomyosin complex in fibroblasts is arranged in a sarcomere-like structure consisting of actin and non-muscle myosin IIa. The motor domain of myosin IIa consists of two heads that can bind to the myosin-binding site on an actin filament. Importantly, during contraction, the myosin IIa molecule progresses in a left-ward "walking" motion towards the barbed end on the myosin-binding site of the actin filament (Beausang *et al.*, 2008; Kovacs *et al.*, 2007), covering around 10–11 nm in each step (**Figure 8B**) (Veigel *et al.*, 1998). In addition, the arrangement of myosin IIa filament is assembled by the tail domain (Melli *et al.*, 2018), forming a myosin IIa bipolar filament in parallel to the actin filaments in the sarcomere-like actomyosin structure (Hu *et al.*, 2017). As a result, the stepwise walking of myosin IIa,

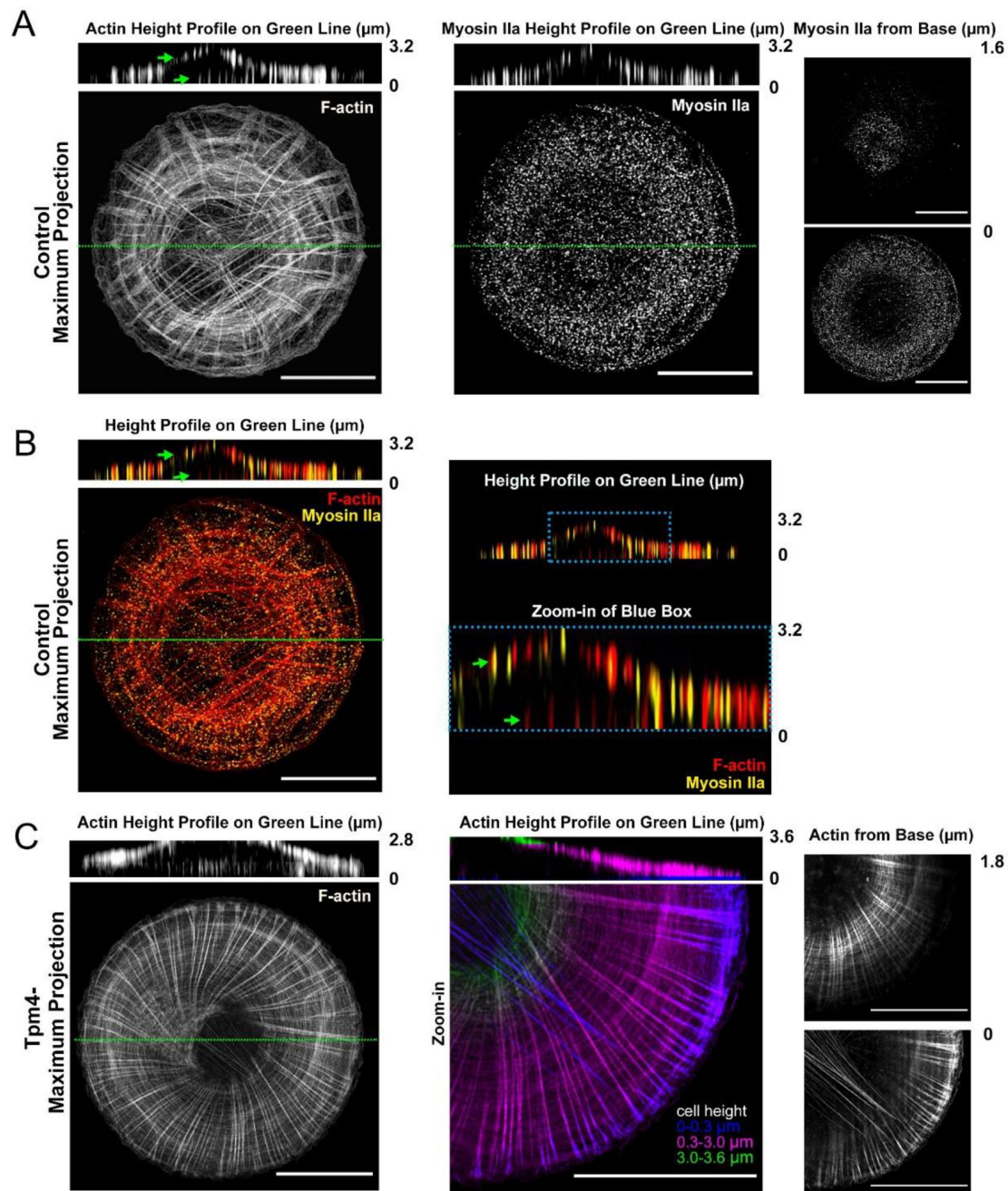


Figure 7.

Actin cytoskeleton and myosin IIa height profile.

(A) The maximum projection of fluorescence staining of F-actin with phalloidin (left) and immunofluorescence staining of myosin IIa (right), with the side view showing the height profile of the cell from the region of interest (top), as indicated by the dashed green line on 2500 μm^2 pattern. Myosin images represent the bottom region and middle region (right). (B) The merged fluorescence images of F-actin (red) and myosin IIa (yellow). The height profile with zoom-in (blue box) at the region of interest (right) is indicated by the dashed green line (left). (C) The maximum projection of F-actin fluorescence staining by phalloidin with Tpm4-silenced HFF-1 cells. The side view (top) shows the height profile of the cell from the region of interest (green line). We zoomed in at the lower right region (bottom) to visualize the structure. The F-actin structure at the bottom focal plane, from 0 to 0.3 μm , is labeled in blue, while the two subsequent sections above are labeled purple and green. The actin image represents the bottom region and middle region (lower right), showing the radial fibers at the base and the transverse arcs at the upper layer, near the cell ceiling. Scale Bar: 20 μm .

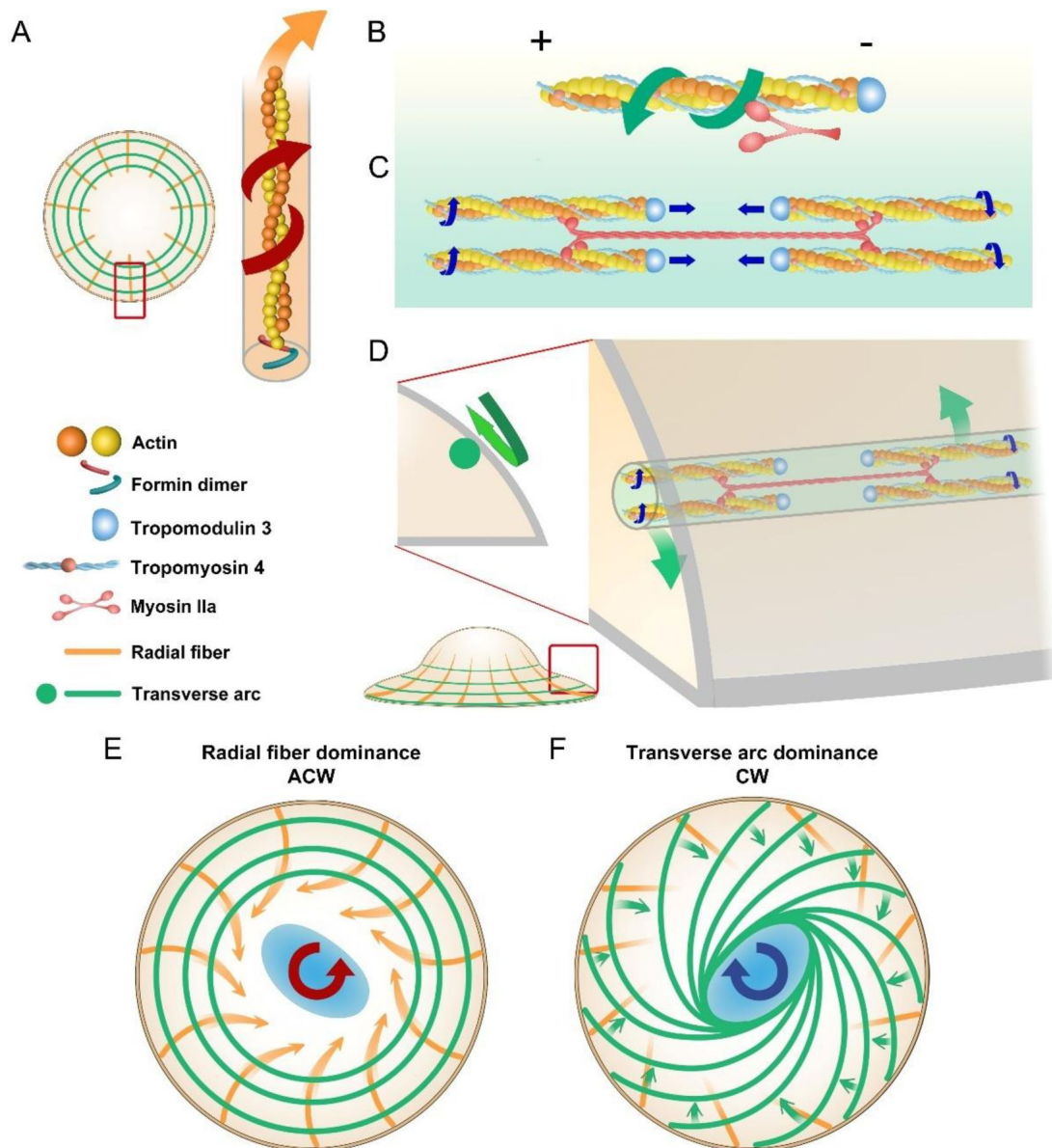


Figure 8.

Actomyosin dynamics of radial fibers and transverse arcs in contribution to cell chirality.

(A) Schematic showing the radial fiber-generating rightward force. As the barbed ends of the right-handed double helix of an actin filament are capped and tethered by formin, incorporation of new actin monomers to the right-handed double helix causes the actin right-screw motion of radial fibers during polymerization. (B) Schematic of the myosin IIa gliding motion on Tpm4-decorated actin filament. Myosin IIa walks leftward from pointed end to barbed end. The green arrow represents the left-handed direction of the myosin IIa head footprint. (C) Schematic showing a unit of sarcomere-like contraction of myosin IIa and actin filament. During contraction, the actin filaments contract to the pointed end through myosin IIa, with a gliding motion creating left-handed twirling of actin filaments to the pointed end. (D) Schematic of transverse arcs producing CW swirling. Transverse arcs are located immediately beneath the cell membrane, away from the substrate. With respect to the cell surface, the contraction of the sarcomere-like unit in the transverse arcs gives rise to tilting. The green arrow demonstrates the rotational force of the sarcomere-like unit in the transverse arc. (E) When radial fiber is dominant, radial fibers with right-screw motion tilt rightward, accompanying retrograde flow along the growing direction of radial fibers and eventually driving an overall ACW rotation of the entire cytoskeleton around the cell nucleus. (F) When transverse arcs are dominant, the tilting of transverse arc precursors causes a CW swirling of transverse arcs that approaches the nucleus during retrograde flow, eventually causing CW rotational wrapping around the nucleus.

which is tethered by the bundled tail, could plausibly drive a left-handed twirling of the actin filaments towards the C-terminal (**Figure 8C** [↗](#); **Video 9** [↗](#)). Thus, the contraction based on left-handed-walking myosin II on actin filaments could serve as a chiral motor with torque dipole. Previous studies indicate that, when near an interface, the frictional force due to relative motion between the torque dipole and the surface, such as the cell cortex, could lead to chiral processes of the cytoskeleton (**Furthauer et al., 2013** [↗](#)). Here, we have consistently identified that transverse arcs are part of the actin filament network near the dorsal surface of cells connection to the extension of radial fibers, consistent with previous results (**Tee et al., 2015** [↗](#)). Thus, the contractile sarcomere-like unit may also act as a torque dipole that “rolls” against the plasma membrane, eventually leading the transverse arcs to tilt and swirl in a CW direction (**Figure 8D** [↗](#), **Video 10** [↗](#)).

We propose that the imbalance between radial fibers and transverse arcs could control the reversal of chirality at the cell level. When radial fibers are dominant, radial fibers with right-screw motion would tilt rightward, accompanied with retrograde flow to establish an overall ACW rotation of cells (**Figure 8E** [↗](#)). In contrast, when the transverse arcs are dominant, the chiral processes of torque dipoles of the transverse arcs would swirl in CW, causing an overall CW rotation (**Figure 8F** [↗](#)). The transmission of chirality from the transverse arcs or the radial fibers to nucleus positioning can be regulated by Transmembrane Actin-Associated Nuclear (TAN) Lines and perinuclear actin caps (**Davidson and Cadot, 2021** [↗](#)), with TAN lines related to retrograde growth and actin caps connected to dorsal or ventral fibers (**Luxton et al., 2010** [↗](#)).

Notably, our proposed mechanism can be a universal, cell-type independent model. In previous results regarding cell alignment (**Wan et al., 2011** [↗](#)), the chirality of C2C12 and hMSCs showed opposite, cell-type dependent chirality. In contrast, we found that C2C12 and hMSCs exhibited similar, cell-type independent chiral reversal. Such a difference can be explained by the cell spreading area when attached to a fibronectin surface in cell culture by staining cell tracker (**Discussion—figure supplement 1** [↗](#)). With different spreading sizes for HFF-1 ($2294.55 \pm 976.5737 \mu\text{m}^2$), C2C12 ($860.8984 \pm 352.624 \mu\text{m}^2$), and hMSCs ($4026.123 \pm 1828.682 \mu\text{m}^2$), the opposite chirality observed previously may simply be due to the cell spreading area on fibronectin coated surfaces falling in the range of CW rotation (C2C12) or ACW rotation (HFF-1 and hMSCs).

In summary, using nucleus rotation, our results indicate the main drivers for chirality, ACW chirality originates from the right-handed axial spinning growth of radial fiber, while CW chirality is driven by the retrograde flow of transverse arcs with CW swirling. An imbalance between these two classes of actin fibers through adjustment of cell spreading area or regulation of the main components of actin fibers (myosin II, α -actinin-1, mDia2, or Tpm4), cell chirality can be reversed. This finding provides a general mechanism of chirality reversal, which has broad implications applicable to different cell types and understanding of tissue morphogenesis.

Methods and Materials

Cell culture

HFF-1 human foreskin fibroblasts (SCRC-1041™, ATCC) were cultured in Dulbecco's Modified Eagle Medium (4 mM L-glutamine, 4500 mg/L glucose, 1 mM sodium pyruvate and 1.5 g/L sodium bicarbonate, Life Technologies) supplemented with 15% fetal bovine serum and 1% penicillin–streptomycin (Life Technologies). Only cell passages from 2 to 10 were used for the experiment. C2C12 mouse myoblasts (CRL-1772™, ATCC) were thawed in P8 and used at P9. They were cultured in Dulbecco's Modified Eagle's Medium (4 mM L-glutamine, 4500 mg/L glucose and 3.7 g/L sodium bicarbonate, Life Technologies) and supplemented with 10% fetal bovine serum and 1% penicillin–

streptomycin (Life Technologies). Human mesenchymal stem cells (hMSCs, PT-2501, Lonza) were also cultured in Dulbecco's Modified Eagle's Medium (Life Technologies) and supplemented with 10% fetal bovine serum and 1% penicillin–streptomycin (Life Technologies).

Nucleus rotation on culture dish

After confluence, the nuclei of HFF-1 cells were stained by applying 2 $\mu\text{g}/\text{ml}$ H33342 trihydrochloride (Sigma-Aldrich) in medium for 30 min. Cells were suspended and reseeded onto a culture dish at a density of 5000 cells per cm^2 and incubated in an incubator at 37 °C for 15 min for cell attachment. After removing the non-attached cells and replenishing with fresh medium, the dish was placed in a live cell incubator on a microscope stage (Chamlide™ TC incubator system, Live Cell Instrument) with 100% humidity and 5% CO₂ in 37C. To record nuclei rotation, fluorescence images of the nuclei and phase contrast images were acquired with an Nikon Eclipse Ti-E microscope at 5 min interval for 4 h. Cell tracking and labeling was performed based on the shortest displacement by comparing the locational information of each cell at the current image and previous time interval, as shown by the trajectory of cell location for 4 h following cell attachment to the culture dish (**Figure 1B** [↗](#)). Automated image segmentation was used to measure the angular velocity of nuclei. In brief, after image binarization, the contour of the cell nuclei was fit by an oval shape to identify its long axis. By calculating the transient angular velocity of the long axis within the time interval, angular velocity was calculated by the angle difference of the nucleus at two consecutive images, which gives the transient angular velocity of each cell of each 5 min interval for the total 4 hour and 30 min time period. By grouping measurement of all cells every 20 min, the probability of ACW or CW rotation was determined. Then, the difference between the ACW/CW percentages was compared based on the projection area and circularity, which was calculated based on cells measured in the Nikon NIS-Elements.

Micropatterning

Photolithography was used to accomplish a series of cell adherent islands with various sizes. Glass slides were treated with piranha solution (sulfuric acid to hydrogen peroxide = 3:1, 100°C) for one hour. After rinsing and drying, the slides were treated with hexamethyldisilazane (HMDS, Sigma-Aldrich) coating in a vapor phase for 5 min. AZ5214 photoresist (AZ Electronic Materials, Luxembourg) was spin-coated on substrates at 3000 rpm and baked at 95 °C for 2 min. The photoresist then underwent UV exposure and was developed using developer (AZ400k: deionized water 1:3) to imprint the desired pattern, followed by plasma treatment to remove the remaining photoresist on the pattern (800 mTorr at 30W). Next, the pattern was coated with fibronectin solution (20 $\mu\text{g}/\text{ml}$, Life Technology) for one hour. Afterward, the remaining photoresist was removed by rinsing in absolute ethanol three times, for 5 min each; substrates were then dried before use. Finally, the substrates were treated with 1% Pluronic F127 for 50 min before cell seeding.

Nucleus rotation on micropatterns with circular or other shapes

HFF-1 fibroblasts and C2C12 myoblasts were seeded at a density of 4000 cells/ cm^2 on circular patterned glass with different sizes ranging from 500 to 2500 μm^2 , while hMSCs were seeded at a density of 1000–2000 cells/ cm^2 on pattern sizes ranging from 1000 to 5000 μm^2 . For micropatterns with other shapes, HFF-1 fibroblasts were seeded at a density of 4000 cells/ cm^2 on equilateral triangle, square or rectangle micropattern of size 750 or 2500 μm^2 . After incubation for 30 min, cells were replenished with fresh growth medium for 3 h. Cells were treated with 2 $\mu\text{g}/\text{mL}$ bisBenzimide H33342 trihydrochloride (Sigma-Aldrich) for 30 min and imaged by Nikon Eclipse Ti-E microscope at every 10 min interval for 2–3 h. The automated image segmentation was used for measuring the nucleus rotation as mentioned previously. The percentages of ACW or CW transient rotation of each cell was determined within the entire duration and averaged among many cells.

Drug treatment

To examine the role of actin in nucleus rotation, cells were treated with 50 nM of Latrunculin A (Sigma-Aldrich), 100 μ M of NSC23766 (Sigma-Aldrich), 2 μ M of A23187 (J&K Scientific), or 10 μ M Y27632 (Alexis).

Analysis of fiber orientation and actin structure on circular pattern

The quantitative analysis of actin fiber orientation was carried out in MATLAB as described in previous studies ([Bao et al., 2020](#)). In brief, an adaptive threshold and Gaussian filter was applied to cropped actin images to generate a binarized image of actin. The central region of actin was removed to highlight the radial fibers and transverse arcs. The core skeleton of actin filament was then obtained by removing lines shorter than 8 pixels. The skeleton was classified as a straight line (the aspect ratio of surrounding ellipse of the line being greater than 4) or a curved line. For straight skeletons, orientation was obtained through the acute angle difference between the long axis of ellipse and the tangential direction to the center. For curved skeletons, the orientation was calculated according to the pixel location on the curved line, determined by the 3-point spline method and the tangential line to the center. Orientation of actin was defined as ACW when orientated from 0° to 90° and CW when orientated from -90° to 0°.

The actin structures can be identified by their characteristics. Radial fibers emerge from the cell boundary and point towards the cell center, which the orientation of radial fibers was defined from -65° to 65°. Transverse arcs are parallel to the cell boundary, the orientation of transverse arc was defined from -90° to -65° and 65° to 90°. With the angle of the filament regarding the cell center, the radial fiber or transverse arc can be classified and counted.

Enucleation of cell

Enucleated HFF-1 cells were prepared according to method stated in the literature ([Degaetano and Schindler, 1987](#)). Cells were plated on glass with fibronectin coating a night before the procedure. The glass was then placed into a 50 ml falcon tube with medium supplemented with 2 μ g/ml of Cytochalasin D (Sigma-Aldrich). The falcon tube was then centrifuged at 10500 $\times$ g at 37 °C for one hour with an Eppendorf 5804R to enucleate cells. Cells were then replenished with fresh medium for recovery. The cells were then trypsinized and reseeded on a circular pattern for 6 hours before fixing. The fixed samples were stained with phalloidin and DAPI to observe the actin structure of enucleated cells.

Plasmid transfection

HFF-1 fibroblasts were seeded one night before the transfection (100,000 cells in a 35 mm dish). We used DNA plasmids, actin-GFP (a gift from Michael Davidson, Addgene plasmid # 56421), mRuby-Lifeact (a gift from Dr. Cheng-Han Yu of the University of Hong Kong), pCDNA_Lifeact-GFP-NLS-mCherry (Lifeact-GFP) (a gift from Olivier Pertz, Addgene plasmid # 69058), pEFmEGFP-mDia2 (a gift from Arthur Alberts, Addgene plasmid # 25407) and pEGFP-N1 alpha-actinin 1 (a gift from Carol Otey, Addgene plasmid # 11908). The OPTIMEM medium (ThermoFisher) and Lipofectamine 3000 Assay (ThermoFisher) were used for transfection. According to manufacturers' suggestions, 500 ng of DNA plasmid was placed in OPTIMEM medium. Following the addition of P3000 Reagent and Lipofectamine 3000 Reagent, the mixture was incubated for 30 min to form lipofectamine complex before being applied to cells. Cells were then incubated with transfection mixture overnight, followed by replenishment with fresh medium for recovery. Next, the transfected cells were reseeded on a micropatterned surface at a density of 4,000 cells/cm². After incubation for 30 min for the cell to spread fully, fresh medium was replenished before imaging.

Optical flow analysis

We used MATLAB's built-in Optical Flow function to estimate the actin flow direction in LatA-treated cells. We used the Farneback method to estimate the motion vectors of every pixel through the comparison of consecutive frames of the video sequence. The sum motion vector of each pixel ($V_x(\text{sum})$, $V_y(\text{sum})$) was calculated by adding the motion vectors with time ($V_x(t, t+1, t+2, \dots)$, $V_y(t, t+1, t+2, \dots)$). The motion direction of each pixel was found by comparing the angle difference between the sum motion vector and the vector of the pixel's location pointed towards the cell center.

Gene silencing

Invitrogen Silencer® Pre-designed siRNA was used to deplete Tropomyosins, Tpm1 (AM51331), Tpm2 (AM51331), Tpm3 (AM16708), Tpm4 (AM16708), and mDia2 (AM16708) (table S1). Transfection on HFF-1 was based on the Lipofectamine 3000 Reagent procedure, as mentioned above, with a final concentration of siRNA at 75 nM. After 24 h of transfection, fresh medium was replenished, and the experiment (nucleus rotation and fluorescence imaging) was performed on day 3 after the addition of transfection complex. Next, cells were treated with 2 $\mu\text{g}/\text{mL}$ bisBenzimide H33342 trihydrochloride for 30 min before seeding on micropatterned circular islands. Incubation was done for 30 min for the cells to spread fully and fresh medium was replenished before imaging.

Fluorescent staining

HFF-1 fibroblasts were seeded on circular islands with sizes of 750 μm^2 and 2500 μm^2 at densities of 4,000 cells/ cm^2 for 30 min. Next, they were replenished with growth medium for 6 h. The cell was then treated with 4% paraformaldehyde (PFA) for 15 min, 0.2% Triton X-100 for 10 min, and Image-iT™ FX (ThermoFisher) signal enhancer for 30 min. Next, depending on the staining target, the cells were applied with Rhodamine Phalloidin (1/40, Life Technology) for one hour, rabbit anti-mDia2 antibodies (1/100, Life Technology), rabbit anti-myosin IIa antibodies (1/100, Cell Signaling), or rabbit anti-Tpm4 antibodies (1/100, Abcam) overnight, followed by application of secondary anti-rabbit antibodies (1/250, Life Technology) for one hour and DAPI staining (300 nM, ThermoFisher) for 5 min. It was then mounted with Fluoromount G (Electron Microscopy Sciences, Inc.) and imaged with a Nikon Eclipse Ti-E microscope. For heatmap distribution of actin, individual images were taken under the same setting (e.g., staining procedure, fluorescent intensity, exposure time, etc.). Next, individual images were stacked and averaged in MATLAB, with reference to the centroids of circular islands, and scaled based on the maximum/minimum intensity of the image. The prerequisite of cells for image stacking was that they had to be fully spread on either 2500 μm^2 or 750 μm^2 circular patterns. Then, the location for image stacking was determined by identifying the center of each cell spread in a perfect circle. Finally, the images were aligned at the center to calculate the averaged intensity to show the distribution heatmap on the circular pattern. For differential heatmaps, the intensity differences were calculated by subtraction between treated and untreated cases and scaled based on the maximum/minimum intensity difference of the image. Each of the individual heatmaps representing the distribution was generated using unique color intensity ranges, which could visualize the protein distribution within cells and variations among samples.

Photobleaching

FRAP experiments were performed using confocal microscope (Zeiss Laser Scanning Microscope LSM 880 NLO with Airyscan). The horseshoe-shaped region of interest (ROI) of the bleaching area was manually drawn. Two successive images of cell were captured prior to photobleaching, using 100% laser power for 40 s, followed by a 380 s recording of the image sequence of the cell with 10 s

time interval. For measurement by photobleaching of radial fiber polymerization and transverse arc translocation velocity, kymographs were performed using an ImageJ plugin to visualize and quantify the movement.

Median intensity analysis

Immunofluorescence imaging of myosin IIa was used to analyze median intensity. The image exhibited a bimodal distribution of pixel intensity, in which the first mode represents the background intensity, and the second mode reflects the myosin IIa expression. The mean intensity of the entire image, which appears in between the two modes, was used as the cut-off intensity to extract the second mode. The second mode was then scanned and the median intensity of the second mode, normalized by the aforementioned cut-off intensity, was then used to represent the myosin IIa expression level. A higher median value indicates a greater intensity of myosin IIa, which suggests more activated myosin IIa.

Pearson's R-value

Fluorescence imaging of myosin IIa and phalloidin was used to analyze the Pearson's R-value. The calculation was carried out using ImageJ with Fiji, the plugin Coloc 2 function. The cell spread on the circular pattern was selected as the region of interest, and the analysis was based on pixel-correlation, which resulted from intensity. The intensity values of the pixels in two images were compared to the corresponding pixel. The Pearson's R-value is calculated by the covariance between two sets of intensity, divided by the product of the standard deviations.

$$r = \frac{n(\sum xy) - (\sum x)(\sum y)}{\sqrt{[n \sum x^2 - (\sum x)^2][n \sum y^2 - (\sum y)^2]}}$$

Lifeact-GFP-or Lifeact-mRuby-transfected Hff-1 cell imaging

Lifeact-GFP-transfected control cells were observed with a Leica SP-8 Confocal Laser Scanning Microscope. A live cell incubation chamber (Leica iRBE) was supplied by Life Imaging Services, with an air heater and CO₂ control. Images were taken at 3 min intervals for 2.25 hours with a 63X oil lens.

Lifeact-mRuby-transfected cells were observed with a Nikon Eclipse Ti2-E microscope with a spinning disk confocal illuminator (LDI-7 Laser Diode Illuminator, 89 North). Images were taken with a 60X oil lens.

Lifeact-GFP-transfected cells treated with LatA were observed with a Nikon Eclipse Ti-E microscope with fluorescent light illuminator. Images were taken with a 40X oil lens.

Western blotting

For gene silencing validation, 75 nM siRNA was transfected for 24 hours and recovered for 48 hours before cell lysis. Proteins were first separated by 4–12 % SDS polyacrylamide gel (ThermoFisher) electrophoresis and transferred to a PVDF membrane (ThermoFisher). The membrane was blocked for one hour before incubation in primary antibodies at 4 °C overnight with the following dilution: GAPDH (1/1000, Cell Signaling), Tpm4 (1/800, Merck), and Diaph3 (1/1000, Abcam). This was followed by HRP-conjugated secondary antibodies (1/2000, Cell Signaling) for one hour at room temperature. Protein expression was detected with Pierce ECL Western Blotting Substrate (ThermoFisher) and the BIO-RAD ChemiDoc MP Imaging System.

Height profile of actin and myosin IIa

In confocal microscopy for the fluorescence image, z-stacks of phalloidin staining of F-actin and immunofluorescence staining of myosin IIa were acquired with a Leica SP-8 Confocal Laser Scanning Microscope with a 63X oil lens and with Leica SP8 LIGHTNING. With a zoom-in function of 2–7 times, the z-stacked image was taken with a step-size of 0.05 to 0.1 μm during the image acquisitions.

Statistical Analysis

Student's t-test was applied to evaluate statistical difference. The confidence level was set to 0.05 for all statistical tests. Statistical significance is symbolized by ns ($p > 0.05$), * ($p \leq 0.05$), ** ($p \text{ value} \leq 0.01$), *** ($p \text{ value} \leq 0.001$), or **** ($p \text{ value} \leq 0.0001$).

Acknowledgements

We thank the Department of Infectious Diseases and Public Health, City University of Hong Kong, and Chinetek Scientific for sharing the Nikon Eclipse Ti2-E microscope with a spinning disk confocal illuminator (LDI-7 Laser Diode Illuminator, 89 North) and for the technical support. This study was supported by the following: Hong Kong Research Grant Council (11217820 and N_CityU119/19), Innovation and Technology Commission (ITS/098/20), The Science, Technology, and Innovation Commission of Shenzhen Municipality (JCYJ20210324134006017), and the City University of Hong Kong (9678242 and 6430620).

Author contributions

Hoi Kwan Kwong, Conceptualization, Methodology, Data curation, Investigation, Writing—original draft, Writing—review and editing; Miu Ling Lam, Supervision, Funding acquisition, Writing—review and editing; Siying Wu, Cho Fan Chung, Jianpeng Wu, Data curation; Lok Ting Chu, King Hoo Lim, Methodology; Hiu Lam Chow, Visualization; Hogi Hartanto, Writing—review and editing; Wengang Liu, Data curation; Kwan Ting Chow, Supervision, Methodology; Ting-Hsuan Chen, Conceptualization, Supervision, Methodology, Data curation, Investigation, Funding acquisition, Writing—original draft, Writing—review and editing.

Additional Declarations

The authors declare no competing interests.

Supplementary Figures

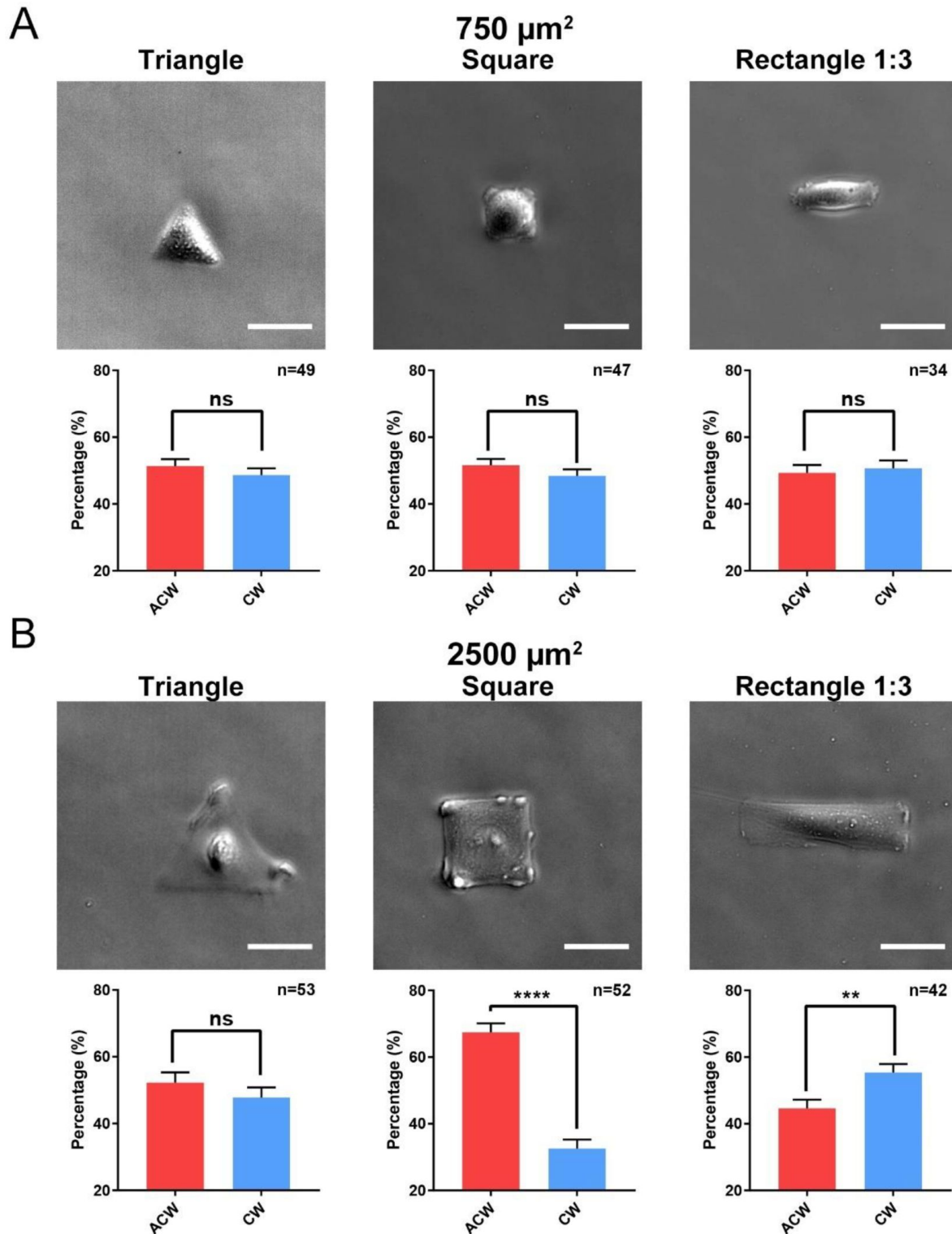


Figure 2—figure supplement 1

Nucleus rotation on micro-islands with varied areas and shapes.

(A-B) Phase contrast images of cells and the probability of ACW/CW rotation with areas of 750 μm^2 (A) and 2500 μm^2 (B). The pattern shapes used are equilateral triangle (circularity = 0.605), square (circularity = 0.785), and 1:3 rectangle (circularity = 0.589). Data presents the mean + SEM. Two-sample equal variance two-tailed t-test was used. Scale bar, 20 μm .

Figure 3—figure supplement 1

Actin orientation analysis of HFF-1 cell.

Fluorescent staining of DAPI-stained nucleus (blue) and F-actin (white) by phalloidin on a $2500\ \mu\text{m}^2$ micro-pattern. Orientation analysis of actin filament on $2500\ \mu\text{m}^2$ island. Mean + SEM. Two-sample equal variance two-tailed t-test.

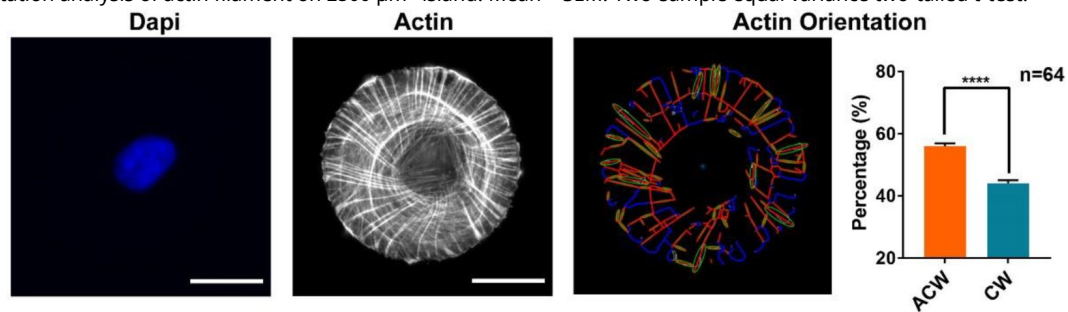
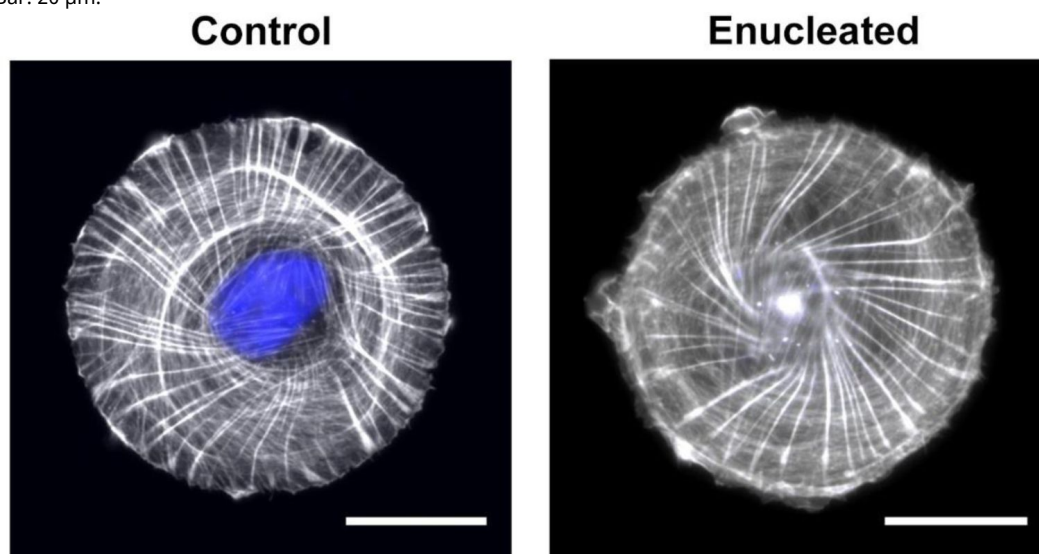


Figure 3—figure supplement 2

Immunofluorescent image of actin distribution of untreated control and enucleated cell on an island with an area of $2500\ \mu\text{m}^2$.

Scale Bar: $20\ \mu\text{m}$.



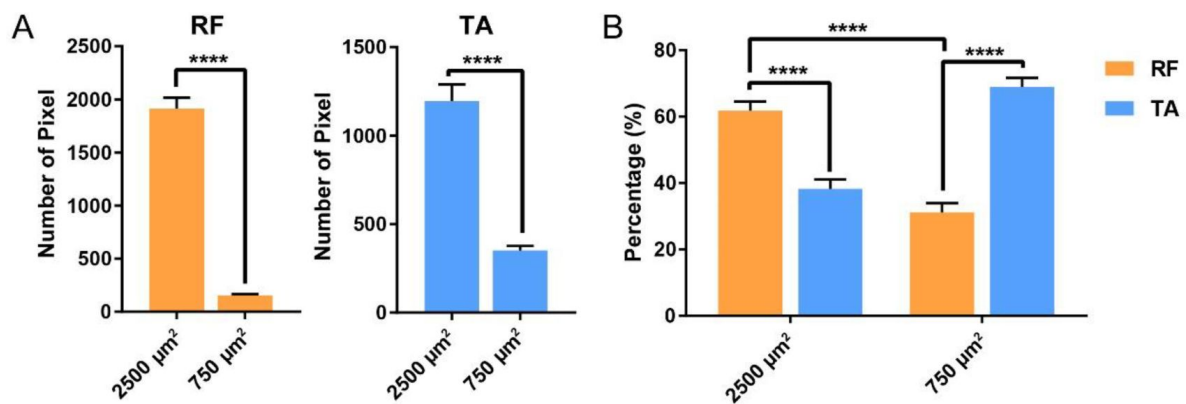


Figure 3—figure supplement 3

Actin structure pixel number and percentage of cell spreading on 2500 μm^2 and 750 μm^2 patterns.

(**A**) Number of pixels of radial fiber (RF) (left) or transverse arc (TA) (right) are compared between the cells on 2500 μm^2 (n = 43) and 750 μm^2 (n = 31) patterns. (**B**) Percentage of RF and TA in cells on 2500 μm^2 (n = 43) and 750 μm^2 (n = 31) patterns. Mean + SEM. Two-sample equal variance two-tailed t-test.

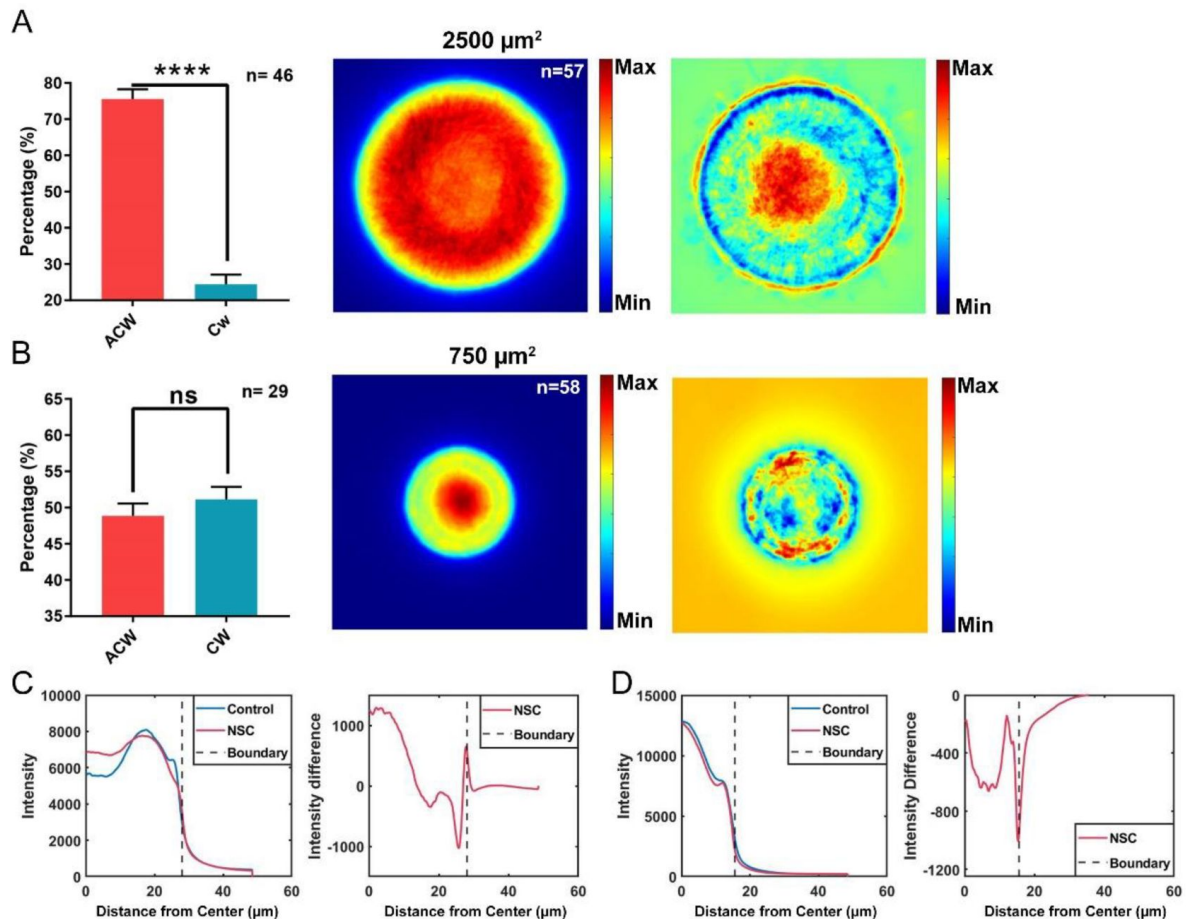


Figure 3—figure supplement 4

Rotational bias of cell nucleus and actin distribution with actin-related inhibitors on HFF-1.

(A-B) Probability of ACW/CW rotation on 2500 μm^2 (A) and 750 μm^2 (B) islands with cytoskeleton inhibitors NSC (left), stacked image of actin showing heatmap distribution (middle) and intensity difference (right) of actin comparing cells treated with NSC to untreated control. Data present the mean + SEM. Two-sample, equal variance two-tailed t-test was used. (C-D) Curves of actin intensity from cell center to boundary (left) and differential actin intensity (right) comparing cells treated with NSC to untreated control on (C) 2500 μm^2 and (D) 750 μm^2 island.

Figure 3—figure supplement 5

Actin percentage of cell with the control and LatA treatment on 2500 μm^2 pattern.

Percentage of RF and TA in cells of untreated control (n = 43, reused data in [Figure 3—figure supplement 3](#)) and cell under LatA treatment (n = 42) on 2500 μm^2 pattern. Mean + SEM. Two-sample equal variance two-tailed t-test.

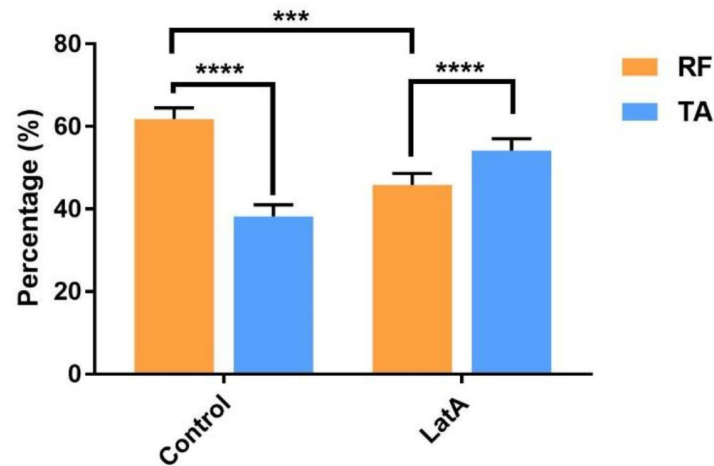


Figure 3—figure supplement 6

Actin percentage of cell with the control and A23 treatment on 750 μm^2 pattern.

Percentage of RF and TA in cells of untreated control (n = 31, reused data in [Figure 3—figure supplement 3](#)) and cell under A23 treatment (n = 29) on 750 μm^2 pattern. Mean + SEM. Two-sample equal variance two-tailed t-test.

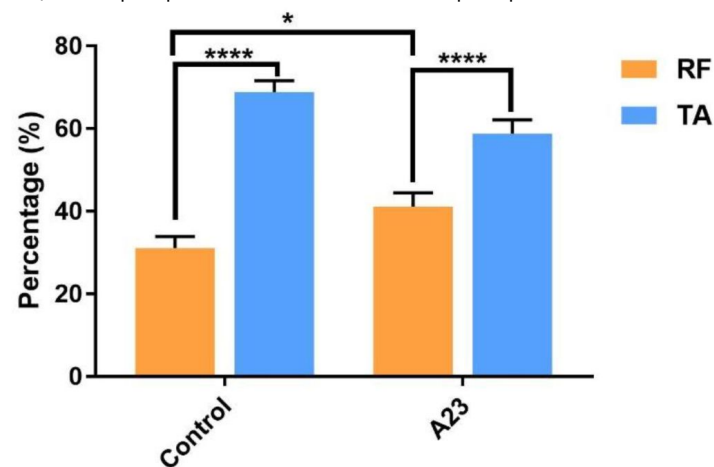


Figure 5—figure supplement 1

Transverse arcs crosslinking protein overexpression.

(A) Transfection of α -actinin-1-GFP showing α -actinin-1 distribution. Scale Bar: 20 μm . (B) Probability of ACW/CW rotation with increscent fluorescence intensity.

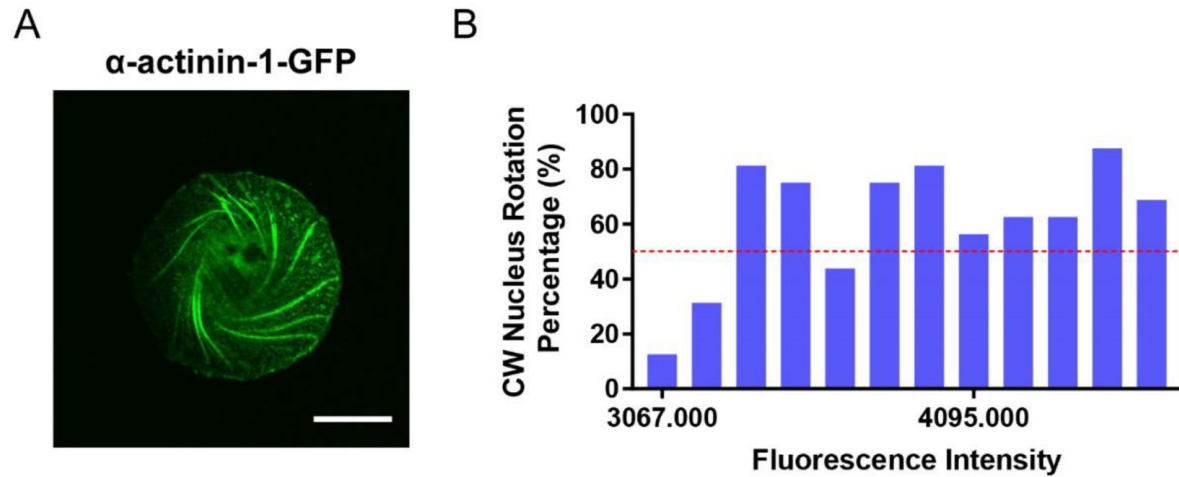


Figure 5—figure supplement 2

Myosin II distribution of Y27632 treatment and control from cell center to the pattern boundary on 2500 μm^2 pattern.

Curves of myosin II intensity from cell center to boundary (left) and differential myosin IIa intensity (right) comparing Y27632 treatment to the control on a 2500 μm^2 pattern.

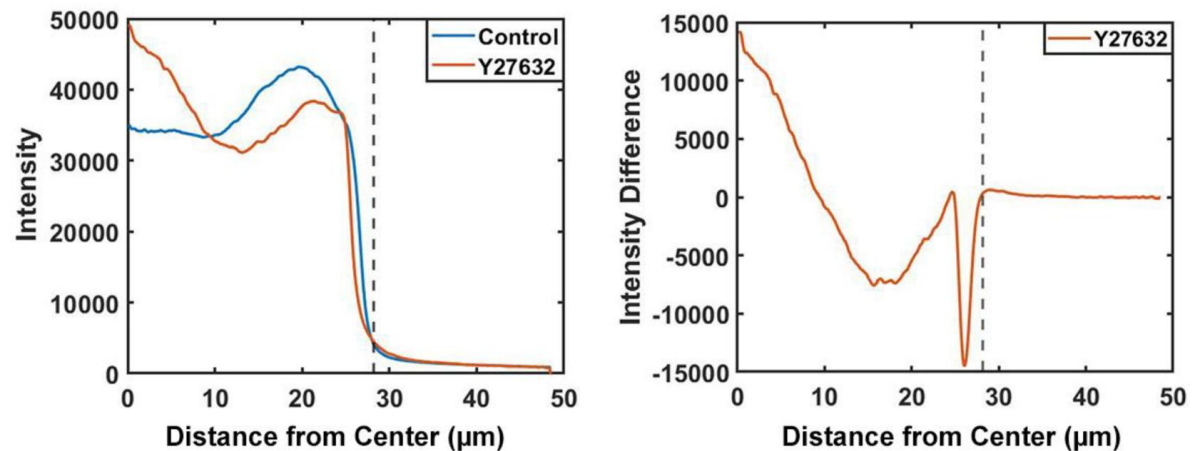


Figure 6—figure supplement 1

Immunofluorescence staining of actin and Tpm4 when spread on a $2500\ \mu\text{m}^2$ pattern.

Scale bar: $20\ \mu\text{m}$.

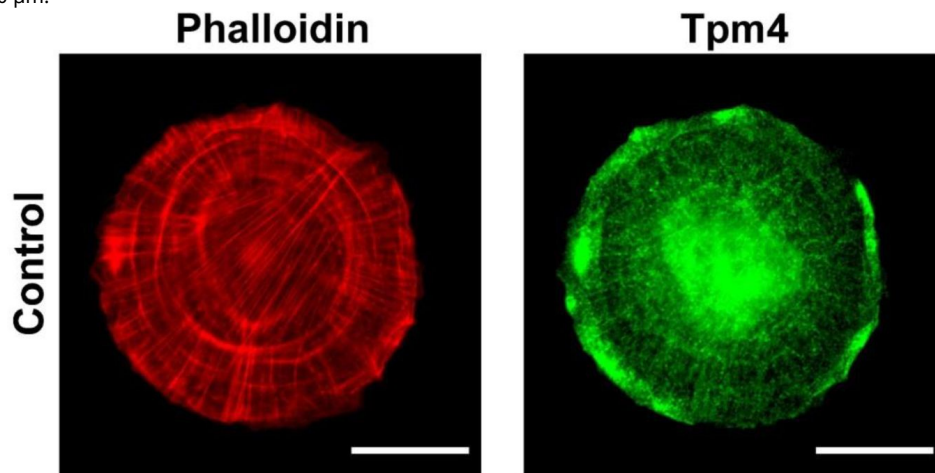
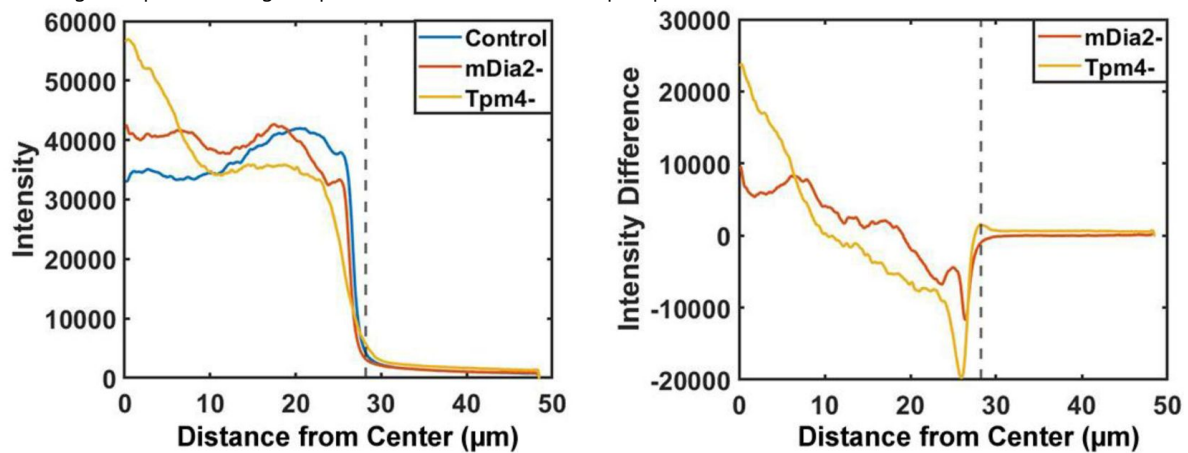


Figure 6—figure supplement 2

Myosin IIa distribution of mDia2 silencing and Tpm4 silencing cells compared to the control from cell center to the pattern boundary on $2500\ \mu\text{m}^2$ pattern.

Curves of myosin IIa intensity of cell from cell center to boundary (left) and differential myosin IIa intensity (right) of mdia2 silencing and Tpm4 silencing compared to the control on a $2500\ \mu\text{m}^2$ pattern.



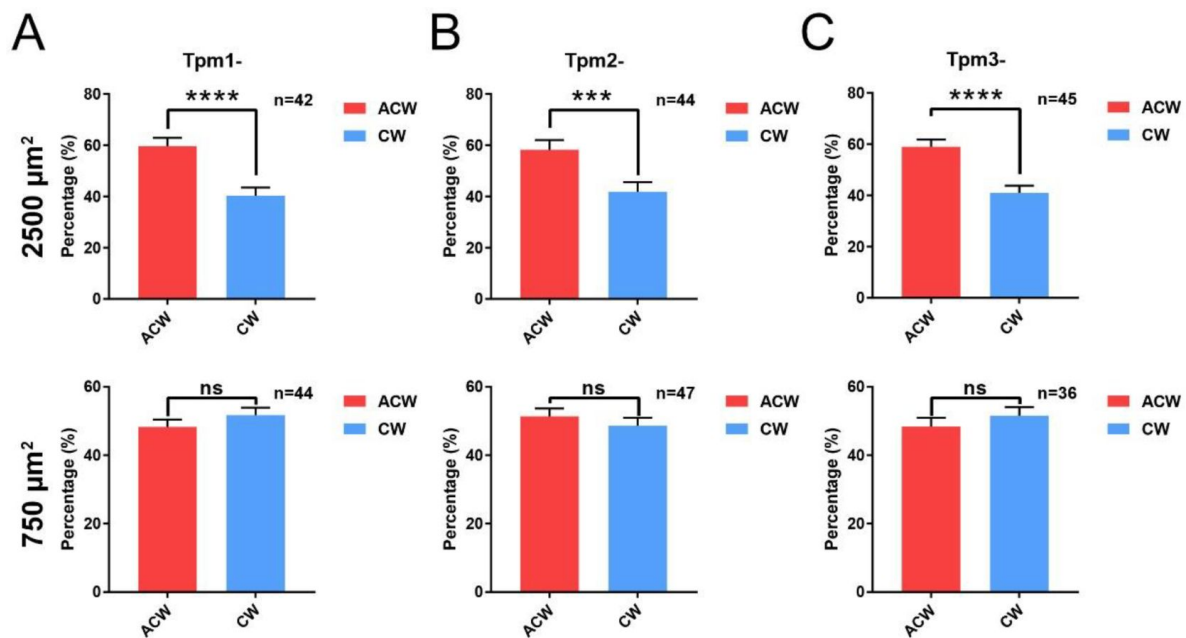


Figure 6—figure supplement 3

Nucleus rotation with different tropomyosin siRNA transfection.

(A-C) Probability of nucleus rotational bias on 2500 μm^2 (upper) and 750 μm^2 (lower) with gene silencing in tropomyosin isoform, Tpm1 (A), Tpm2 (B) and Tpm3 (C). Data present the mean + SEM. Two-sample equal variance two-tailed t-test was used.

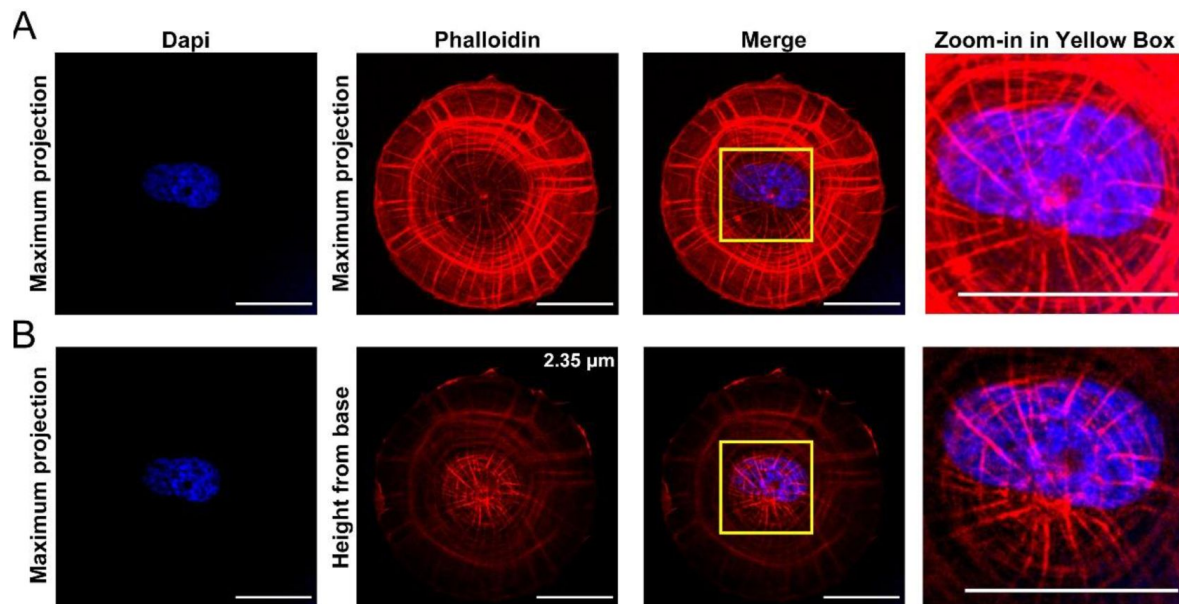
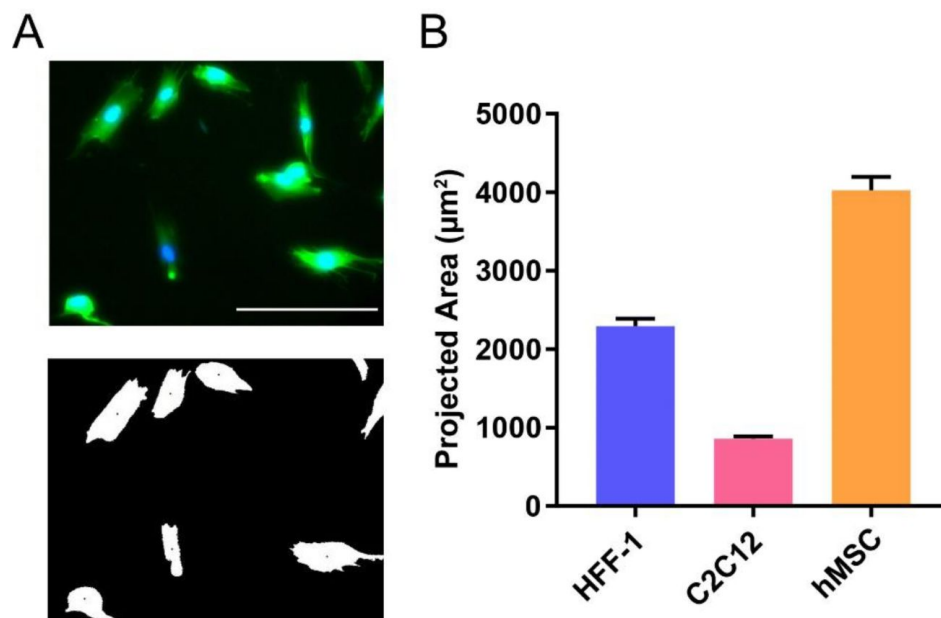


Figure 7—figure supplement 1

Actin structure by z-stack of confocal microscopy.

(A) Maximum projection of DAPI-stained nucleus (blue) and phalloidin-stained F-actin (red). (B) Maximum projection of DAPI-stained nucleus (blue) and phalloidin image at 2.35 μm from the bottom of the z-stacked microscopy. The merged image of phalloidin and DAPI shows transverse arcs are present on top of the nucleus with conjunction to a perinuclear actin cap. Scale bar: 20 μm .



Discussion—figure supplement 1

Natural spreading size of cell.

(A) Cell culture staining with cell tracker (green) and DAPI (blue) (upper) and the binarized area of cell (lower). Scale bar, 200 μm . (B) The projected area measurement of HFF-1 (2294.55 $\pm$ 93.1125 μm^2 , n = 110), C2C12 (860.8984 $\pm$ 26.7324 μm^2 , n = 175) and hMSCs (4026.123 $\pm$ 172.0287 μm^2 , n = 113). Data presents the mean + SEM.

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Editors

Reviewing Editor

Alphee Michelot

Institut de Biologie du Développement, Marseille, France

Senior Editor

Merritt Maduke

Stanford University, Stanford, United States of America

Reviewer #3 (Public Review):

Summary:

In this work, the authors plate different type of cells on circular micropatterns and question how the organization and dynamics of the actin cytoskeleton correlate with particular actin chiral properties and rotational direction of the nucleus. The observe that cell spreading on large patterns correlates with the emergence of anti-clockwise rotations (ACW), while spreading on small patterns leads preferentially to clockwise rotations (CW). ACW originate, as previously demonstrated, from the polymerization of radial fibers, while clockwise rotations (CW) are observed when radial fibers are disorganized or absent and when transverse arcs take over to power CW rotations. These data are supported by a large number

of observations and use of multiple drugs lead to observations that are consistent with the proposed model.

Strengths:

This is a beautiful work in which the authors rely on a large number of high-quality microscopic observations and use a full arsenal of drugs to test their model as thoroughly as possible.

This study examines the influence of multiple actin networks. This is a challenging task in that the assembly and dynamics of different actin networks are interdependent, making it difficult to unambiguously analyze the importance of any specific network.

<https://doi.org/10.7554/eLife.92632.2.sa1>

Author response:

The following is the authors' response to the original reviews.

Preliminary note from the Reviewing Editor:

The evaluations of the two Reviewers are provided for your information. As you can see, their opinions are very different.

Reviewer #1 is very harsh in his/her evaluation. Clearly, we don't expect you to be able to affect one type of actin network without affecting the other, but rather to change the balance between the two. However, he/she also raises some valid points, in particular that more rationale should be added for the perturbations (also mentioned by Reviewer #2). Both Reviewers have also excellent suggestions for improving the presentation of the data.

We sincerely appreciate your and the reviewers' suggestions. The comments are amended accordingly.

On another point, I was surprised when reading your manuscript that a molecular description of chirality change in cells is presented as a completely new one. Alexander Bershadsky's group has identified several factors (including alpha-actinin) as important regulators of the direction of chirality. The articles are cited, but these important results are not specifically mentioned. Highlighting them would not call into question the importance of your work, but might even provide additional arguments for your model.

We appreciate the editor's comment. Alexander Bershadsky's group has done marvelous work in cell chirality. They introduced the stair-stepping and screw theory, which suggested how radial fiber polymerization generates ACW force and drives the actin cytoskeleton into the ACW pattern. Moreover, they have identified chiral regulators like alpha-actinin 1, mDia1, capZB, and profilin 1, which can reverse or neutralize the chiral expression.

It is worth noting that Bershadsky's group primarily focuses on radial fibers. In our manuscript, instead, we primarily focused on the contractile unit in the transverse arcs and CW chirality in our investigation. Our manuscript incorporates our findings in the transverse arcs and the radial fibers theory by Bershadsky's group into the chirality balance hypothesis, providing a more comprehensive understanding of the chirality expression.

We have included relevant articles from Alexander Bershadsky's group, we agree that highlighting these important results of chiral regulators would further strengthen our manuscript. The manuscript was revised as follows:

“ACW chirality can be explained by the right-handed axial spinning of radial fibers during polymerization, i.e. ‘stair-stepping’ mode proposed by Tee et al. (Tee et al. 2015) (Figure 8A; Video 4). As actin filament is formed in a right-handed double helix, it possesses an intrinsic chiral nature. During the polymerization of radial fiber, the barbed end capped by formin at focal adhesion was found to recruit new actin monomers to the filament. The tethering by formin during the recruitment of actin monomers contributes to the right-handed tilting of radial fibers, leading to ACW rotation. Supporting this model, Jalal et al. (Jalal et al. 2019) showed that the silencing of mDia1, capZB, and profilin 1 would abolish the ACW chiral expression or reverse the chirality into CW direction. Specifically, the silencing of mDia1, capZB or profilin-1 would attenuate the recruitment of actin monomer into the radial fiber, with mDia1 acting as the nucleator of actin filament (Tsuji et al. 2002), CapZB promoting actin polymerization as capping protein (Mukherjee et al. 2016), and profilin-1 facilitating ATP-bound G-actin to the barbed ends (Haarer and Brown 1990; Witke 2004). The silencing resulted in a decrease in the elongation velocity of radial fiber, driving the cell into neutral or CW chirality. These results support that our findings that reduction of radial fiber elongation can invert the balance of chirality expression, changing the ACW-expressing cell into a neutral or CW-expressing cell.”

By incorporating their findings into our revision and discussion, we provide additional support for our radial fiber-transverse arc balance model for chirality expression. The revision is made on pages 8 to 9, 13, lines 253 to 256, 284, 312 to 313, 443, 449 to 459.

Public Reviews:

Reviewer #1 (Public Review):

Summary:

Kwong et al. present evidence that two actin-filament based cytoskeletal structures regulate the clockwise and anticlockwise rotation of the cytoplasm. These claims are based on experiments using cells plated on micropatterned substrates (circles). Previous reports have shown that the actomyosin network that forms on the dorsal surface of a cell plated on a circle drives a rotational or swirling pattern of movement in the cytoplasm. This actin network is composed of a combination of non-contractile radial stress fibers (AKA dorsal stress fibers) which are mechanically coupled to contractile transverse actin arcs (AKA actin arcs). The authors claim that directionality of the rotation of the cytoplasm (i.e., clockwise or anticlockwise) depends on either the actin arcs or radial fibers, respectively. While this would be interesting, the authors are not able to remove either actin-based network without affecting the other. This is not surprising, as it is likely that the radial fibers require the arcs to elongate them, and the arcs require the radial fibers to stop them from collapsing. As such, it is difficult to make simple interpretations such as the clockwise bias is driven by the arcs and anticlockwise bias is driven by the radial fibers.

Weaknesses:

(1) There are also multiple problems with how the data is displayed and interpreted. First, it is difficult to compare the experimental data with the controls as the authors do not include control images in several of the figures. For example, Figure 6 has images showing myosin IIA distribution, but Figure 5 has the control image. Each figure needs to show controls. Otherwise, it will be difficult for the reader to understand the differences in localization of the proteins shown. This could be accomplished by either adding different control examples or by combining figures.

We appreciate the reviewer's comment. We agree with the reviewer that it is difficult to compare our results in the current arrangement. The controls are included in the new Figure 6.

(2) It is important that the authors should label the range of gray values of the heat maps shown. It is difficult to know how these maps were created. I could not find a description in the methods, nor have previous papers laid out a standardized way of doing it. As such, the reader needs some indication as to whether the maps showing different cells were created the same and show the same range of gray levels. In general, heat maps showing the same protein should have identical gray levels. The authors already show color bars next to the heat maps indicating the range of colors used. It should be a simple fix to label the minimum (blue on the color bar) and the maximum (red on the color bar) gray levels on these color bars. The profiles of actin shown in Figure 3 and Figure 3-figure supplement 3 were useful for interpreting the distribution of actin filaments. Why did not the authors show the same for the myosin IIa distributions?

We appreciate the reviewer's comment. For generating the distribution heatmap, the images were taken under the same setting (e.g., fluorescent staining procedure, excitation intensity, or exposure time). The prerequisite of cells for image stacking was that they had to be fully spread on either 2500 μm^2 or 750 μm^2 circular patterns. Then, the location for image stacking was determined by identifying the center of each cell spread in a perfect circle. Finally, the images were aligned at the cell center to calculate the averaged intensity to show the distribution heatmap on the circular pattern. Revision is made on pages 19 to 20, lines 668 to 677.

It is important to note that the individual heatmaps represent the normalized distribution generated using unique color intensity ranges. This approach was chosen to emphasize the proportional distribution of protein within cells and its variations among samples, especially for samples with generally lower expression levels. Additionally, a differential heatmap with its own range was employed to demonstrate the normalized differences compared to the control sample. Furthermore, to provide additional insight, we plotted the intensity profile of the same protein with the same size for comparative analysis. Revision is made on pages 20, lines 679 to 682.

The labels of the heatmap are included to show the intensity in the revised Figure 3, Figure 5, Figure 6, and Figure 3—figure supplement 4.

To better illustrate the myosin IIa distribution, the myosin intensity profiles were plotted for Y27 treatment and gene silencing. The figures are included as Figure 5—figure supplement 2 and Figure 6—figure supplement 2. Revisions are made on pages 10, lines 332 to 334 and pages 11, lines 377 to 379.

(3) Line 189 "This absence of radial fibers is unexpected". The authors should clarify what they mean by this statement. The claim that the cell in Figure 3B has reduced radial stress fiber is not supported by the data shown. Every actin structure in this cell is reduced compared to the cell on the larger micropattern in Figure 3A. It is unclear if the radial stress fibers are reduced more than the arcs. Are the authors referring to radial fiber elongation?

We appreciate the reviewer's comment. We calculated the structures' pixel number and the percentage in the image to better illustrate the reduction of radial fiber or transverse arc. As radial fibers emerge from the cell boundary and point towards the cell center and the transverse arcs are parallel to the cell edge, the actin filament can be identified by their angle

with respect to the cell center. We found that the pixel number of radial fiber is greatly reduced by 91.98 % on 750 μm^2 compared to the 2500 μm^2 pattern, while the pixel number of transverse arc is reduced by 70.58 % (Figure 3- figure supplement 3A). Additionally, we compared the percentage of actin structures on different pattern sizes (Figure 3- figure supplement 3B). On 2500 μm^2 pattern, the percentage of radial fiber in the actin structure is 61.76 ± 2.77 %, but it only accounts for 31.13 ± 2.76 % while on 750 μm^2 pattern. These results provide evidence of the structural reduction on a smaller pattern.

Regarding the radial fiber elongation, we only discussed the reduction of radial fiber on 750 μm^2 compared to the 2500 μm^2 pattern in this part. For more understanding of the radial fiber contribution to chirality, we compared the radial fiber elongation rate in the LatA treatment and control on 2500 μm^2 pattern (Figure 4). This result suggests the potential role of radial fiber in cell chirality. Revisions are made on page 6, lines 186 to 194; pages 17 to 18, 601 to 606; and the new Figure 3- figure supplement 3.

(4) The choice of the small molecule inhibitors used in this study is difficult to understand, and their results are also confusing. For example, sequestering G actin with Latrunculin A is a complicated experiment. The authors use a relatively low concentration (50 nM) and show that actin filament-based structures are reduced and there are more in the center of the cell than in controls (Figure 3E). What was the logic of choosing this concentration?

We appreciate the reviewer's comment. The concentration of drugs was selected based on literatures and their known effects on actin arrangement or chiral expression.

For example, Latrunculin A was used at 50 nM concentration, which has been proven effective in reversing the chirality at or below 50 nM (Bao et al., 2020; Chin et al., 2018; Kwong et al., 2019; Wan et al., 2011). Similarly, the 2 μM A23187 treatment concentration was selected to initiate the actin remodeling (Shao et al., 2015). Furthermore, NSC23677 at 100 μM was found to efficiently inhibit the Rac1 activation and resulted in a distinct change in actin structure (Chen et al., 2011; Gao et al., 2004), enhancing ACW chiral expression. The revision is made on pages 6 to 7, lines 202 to 211.

(5) Using a small molecule that binds the barbed end (e.g., cytochalasin) could conceivably be used to selectively remove longer actin filaments, which the radial fibers have compared to the lamellipodia and the transverse arcs. The authors should articulate how the actin cytoskeleton is being changed by latrunculin treatment and the impact on chirality. Is it just that the radial stress fibers are not elongating? There seems to be more radial stress fibers than in controls, rather than an absence of radial stress fibers.

We appreciate the reviewer's comment. Our results showed Latrunculin A treatment reversed the cell chirality. To compare the amount of radial fiber and transverse arc, we calculated the structures' pixel percentage. We found that, the percentage of radial fibers pixel with LatA treatment was reduced compared to that of the control, while the percentage of transverse arcs pixel increased (Figure 3— figure supplement 5). This result suggests that radial fibers are inhibited under Latrunculin A treatment.

Furthermore, the elongation rate of radial fibers is reduced by Latrunculin A treatment (Figure 4). This result, along with the reduction of radial fiber percentage under Latrunculin A treatment suggests the significant impact of radial fiber on the ACW chirality. Revisions are made on pages 7 to 8, lines 244 to 250 and the new Figure 3— figure supplement 5 and Figure 3— figure supplement 6.

(6) Similar problems arise from the other small molecules as well. LPA has more effects than simply activating RhoA. Additionally, many of the quantifiable effects of LPA

treatment are apparent only after the cells are serum starved, which does not seem to be the case here.

We appreciate the reviewer's comment. The reviewer mentioned that the quantifiable effects of LPA treatments were seen after the cells were serum-starved. LPA is known to be a serum component and has an affinity to albumin in serum (Moolenaar, 1995). Serum starvation is often employed to better observe the effects of LPA by comparing conditions with and without LPA. We agree with the reviewer that the effect of LPA cannot be fully seen under the current setting. Based on the reviewer's comment and after careful consideration, we have decided to remove the data related to LPA from our manuscript. Revisions are made on pages 6 to 7, 17 and Figure 3—figure supplement 4.

(7) Furthermore, inhibiting ROCK with, Y-27632, effects myosin light chain phosphorylation and is not specific to myosin IIA. Are the two other myosin II paralogs expressed in these cells (myosin IIB and myosin IIC)? If so, the authors' statements about this experiment should refer to myosin II not myosin IIA.

We appreciate the reviewer's comment. We agree that ensuring accuracy and clarity in our statements is important. The terminology is revised to myosin II regarding the Y27632 experiment for a more concise description. Revision is made on pages 9 to 10 and 29, lines 317 to 341, 845 and 848.

(8) None of the uses of the small molecules above have supporting data using a different experimental method. For example, backing up the LPA experiment by perturbing RhoA tho.

We appreciate the reviewer's comment. After careful consideration, we have decided to remove the data related to LPA from our manuscript. Revisions are made on pages 6 to 7, 17 and Figure 3—figure supplement 4.

(9) The use of SMIFH2 as a "formin inhibitor" is also problematic. SMIFH2 also inhibits myosin II contractility, making interpreting its effects on cells difficult to impossible. The authors present data of mDia2 knockdown, which would be a good control for this SMIFH2.

We appreciate the reviewer's comment. We agree that there is potential interference of SMIFH2 with myosin II contractility, which could introduce confounding factors to the results. Based on your comment and further consideration, we have decided to remove the data related to SMIFH2 from our manuscript. Revisions are made on pages 6 to 7, 10, 17 and Figure 3—figure supplement 4.

(10) However, the authors claim that mDia2 "typically nucleates tropomyosin-decorated actin filaments, which recruit myosin II and anneal endwise with α -actinin- crosslinked actin filaments."

There is no reference to this statement and the authors own data shows that both arcs and radial fibers are reduced by mDia2 knockdown. Overall, the formin data does not support the conclusions the authors report.

We appreciate the reviewer's comment. We apologize for the lack of citation for this claim. To address this, we have added a reference to support this claim in the revised manuscript (Tojkander et al., 2011). Revision is made on page 10, line 345 to 347.

Regarding the actin structure of mDia2 gene silencing, our results showed that myosin II was disassociated from the actin filament compared to the control. At the same time, there is no

considerable differences in the actin structure of radial fibers and transverse arcs between the mDia2 gene silencing and the control.

(11) *The data in Figure 7 does not support the conclusion that myosin IIa is exclusively on top of the cell. There are clear ventral stress fibers in A (actin) that have myosin IIa localization. The authors simply chose to not draw a line over them to create a height profile.*

We appreciate the reviewer's comment. To better illustrate myosin IIa distribution in a cell, we have included a video showing the myosin IIa staining from the base to the top of the cell (Video 7). At the cell base, the intensity of myosin IIa is relatively low at the center. However, when the focal plane elevates, we can clearly see the myosin II localizes near the top of the cell (Figure 7B and Video 7). Revision is made on page 12, lines 421 to 424, and the new Video 7.

Reviewer #2 (Public Review):

Summary:

Chirality of cells, organs, and organisms can stem from the chiral asymmetry of proteins and polymers at a much smaller lengthscale. The intrinsic chirality of actin filaments (F-actin) is implicated in the chiral arrangement and movement of cellular structures including F-actin-based bundles and the nucleus. It is unknown how opposite chiralities can be observed when the chirality of F-actin is invariant. Kwong, Chen, and co-authors explored this problem by studying chiral cell-scale structures in adherent mammalian cultured cells. They controlled the size of adhesive patches, and examined chirality at different timepoints. They made various molecular perturbations and used several quantitative assays. They showed that forces exerted by antiparallel actomyosin bundles on parallel radial bundles are responsible for the chirality of the actomyosin network at the cell scale.

Strengths:

Whereas previously, most effort has been put into understanding radial bundles, this study makes an important distinction that transverse or circumferential bundles are made of antiparallel actomyosin arrays. A minor point that was nice for the paper to make is that between the co-existing chirality of nuclear rotation and radial bundle tilt, it is the F-actin driving nuclear rotation and not the other way around. The paper is clearly written.

Weaknesses:

The paper could benefit from grammatical editing. Once the following Major and Minor points are addressed, which may not require any further experimentation and does not entail additional conditions, this manuscript would be appropriate for publication in eLife.

Recommendations for the authors:

Reviewer #2 (Recommendations For The Authors):

Major:

(1) *The binary classification of cells as exhibiting clockwise or anticlockwise F-actin structures does not capture the instances where there is very little chirality, such as in the mDia2-depleted cells on small patches (Figure 6B). Such reports of cell chirality throughout the cell population need to be reported as the average angle of F-actin*

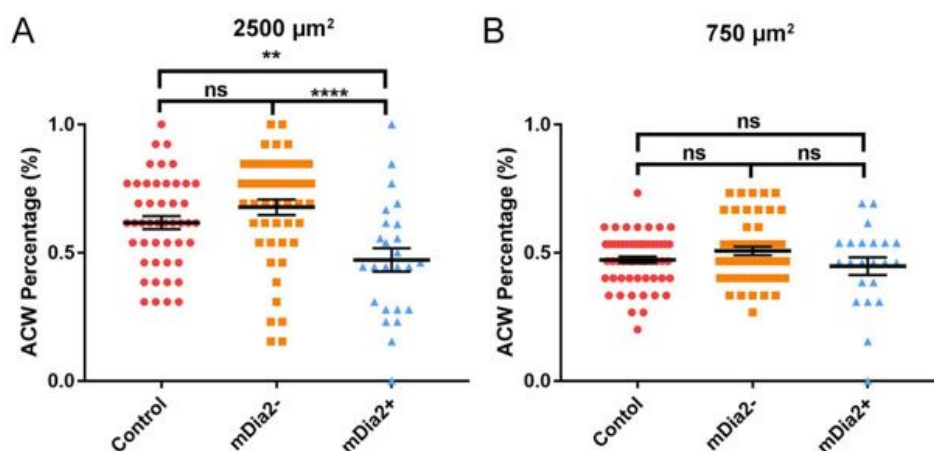
structures on a per cell basis as a rose plot or scatter plot of angle. These changes to cell-scoring and data display will be important to discern between conditions where chirality is random (50% CW, 50% ACW) from conditions where chirality is low (radial bundles are radial and transverse arcs are circumferential).

We appreciate the reviewer's comment. We apologize if we did not convey our analysis method clearly enough. Throughout the manuscript, unless mentioned otherwise, the chirality analysis was based on the chiral nucleus rotation within a period of observation. The only exception is the F-actin structure chirality, in Figure 3—figure supplement 1, which we analyzed the angle of radial fiber of the control cell on 2500 μm^2 . It was described on pages 5 to 6, lines 169-172, and the method section "Analysis of fiber orientation and actin structure on circular pattern" on page 17.

Based on the feedback, we attempted to use a scatter plot to present the mDia2 overexpression and silencing to show the randomness of the result. However, because scatter plots primarily focus on visualizing the distribution, they become cluttered and visually overwhelming, as shown below.

Author response image 1.

(A) Percentage of ACW nucleus rotational bias on 2500 μm^2 with untreated control (reused data from Figure 3D, $n = 57$), mDia2 silencing ($n = 48$), and overexpression ($n = 25$). (B) Probability of ACW/CW rotation on 750 μm^2 pattern with untreated control (reused data from Figure 3E, $n = 34$), mDia2 silencing ($n = 53$), and overexpressing ($n = 22$). Mean $\pm$ SEM. Two-sample equal variance two-tailed t-test.



Therefore, in our manuscript, the presentation primarily used a column bar chart with statistical analysis, the Student T-test. The column bar chart makes it easier to understand and compare values. In brief, the Student T-test is commonly used to evaluate whether the means between the two groups are significantly different, assuming equal variance. As such, the Student T-test is able to discern the randomness of the chirality.

(2) The authors need to discuss the likely nucleator of F-actin in the radial bundles, since it is apparently not mDia2 in these cells.

We appreciate the reviewer's comment. In our manuscript, we originally focused on mDia2 and Tpm4 as they are the transverse arc nucleator and the mediator of myosin II motion. However, we agree with the reviewer that discussing the radial fiber nucleator would

provide more insight into radial fiber polymerization in ACW chirality and improve the completeness of the story.

Radial fiber polymerizes at the focal adhesion. Several proteins are involved in actin nucleation or stress fiber formation at the focal adhesion, such as Arp2/3 complex (Serrels et al., 2007), Ena/VASP (Applewhite et al., 2007; Gateva et al., 2014), and formins (Dettenhofer et al., 2008; Sahasrabudhe et al., 2016; Tsuji et al., 2002), etc. Within the formin family, mDia1 is the likely nucleator of F-actin in the radial bundle. The presence of mDia1 facilitates the elongation of actin bundles at focal adhesion (Hotulainen and Lappalainen, 2006). Studies by Jalal, et al (2019) (Jalal et al., 2019) and Tee, et al (2023) (Tee et al., 2023), have demonstrated the silencing of mDia1 abolished the ACW actin expression. Silencing of other nucleation proteins like Arp2/3 complex or Ena/VASP would only reduce the ACW actin expression without abolishing it.

Based on these findings, the attenuation of radial fiber elongation would abolish the ACW chiral expression, providing more support for our model in explaining chirality expression.

This part is incorporated into the Discussion. The revision is made on page 13, lines 443, 449 to 459.

Minor:

(1) In the introduction, additional observations of handedness reversal need to be referenced (line 79), including Schonegg, Hyman, and Wood 2014 and Zaatri, Perry, and Maddox 2021.

We appreciate the reviewer's comment. The observations of handedness reversal references are cited on page 3, line 78 to 79.

(2) For clarity of logic, the authors should share the rationale for choosing, and results from administering, the collection of compounds as presented in Figure 3 one at a time instead of as a list.

We appreciate the reviewer's comment. The concentration of drugs was determined based on existing literature and their known outcomes on actin arrangement or chiral expression.

To elucidate, the use of Latrunculin A was based on previous studies, which have demonstrated to reverse the chirality at or below 50 nM (Bao et al., 2020; Chin et al., 2018; Kwong et al., 2019; Wan et al., 2011). Because inhibiting F-actin assembly can lead to the expression of CW chirality, we hypothesized that the opposite treatment might enhance ACW chirality. Therefore, we chose A23187 treatment with 2 μ M concentration as it could initiate the actin remodeling and stress fiber formation (Shao et al., 2015).

Furthermore, in the attempt to replicate the reversal of chirality by inhibiting F-actin assembly through other pathways, we explored NSC23677 at 100 μ M, which was found to inhibit the Rac1 activation (Chen et al., 2011; Gao et al., 2004) and reduce cortical F-actin assembly (Head et al., 2003). However, it failed to reverse the chirality but enhanced the ACW chirality of the cell.

We carefully selected the drugs and the applied concentration to investigate various pathways and mechanisms that influence actin arrangement and might affect the chiral expression. We believe that this clarification strengthens the rationale behind our choice of drug. The revision is made on pages 6 to 7, lines 202 to 211.

(3) "Image stacking" isn't a common term to this referee. Its first appearance in the main text (line 183) should be accompanied with a call-out to the Methods section. The authors

could consider referring to this approach more directly. Related issue: Image stacking fails to report the prominent enrichment of F-actin at the very cell periphery (see Figure 3 A and F) except for with images of cells on small islands (Figure 3H). Since this data display approach seems to be adding the intensity from all images together, and since cells on circular adhesive patches are relatively radially symmetric, it is unclear how to align cells, but perhaps cells could be aligned based on a slight asymmetry such as the peripheral location with highest F-actin intensity or the apparent location of the centrosome.

We appreciate the reviewer's comment. We fully acknowledge the uncommon use of "image stacking" and the insufficient description of image stacking under the Method section. First, we have added a call-out to the Methods section at its first appearance (Page 6, Lines 182 to 183). The method of image stacking is as follows. During generating the distribution heatmap, the images were taken under the same setting (e.g., staining procedure, fluorescent intensity, exposure time, etc.). The prerequisite of cells to be included in image stacking was that they had to be fully spread on either 2500 μm^2 or 750 μm^2 circular patterns. Then, the consistent position for image stacking could be found by identifying the center of each cell spreading in a perfect circle. Finally, the images were aligned at the center to calculate the averaged intensity to show the distribution heatmap on the circular pattern.

We agree with the reviewer that our image alignment and stacking are based on cells that are radially symmetric. As such, the intensity distribution of stacked image is to compare the difference of F-actin along the radial direction. Revision is made on page 19, lines 668 to 682.

(4) The authors need to be consistent with wording about chirality, avoiding "right" and left (e.g. lines 245-6) since if the cell periphery were oriented differently in the cropped view, the tilt would be a different direction side-to-side but the same chirality. This section is confusing since the peripheral radial bundles are quite radial, and the inner ones are pointing from upper left to lower right, pointing (to the right) more downward over time, rather than more right-ward, in the cropped images.

We appreciate the reviewer's comment. We apologize for the confusion caused by our description of the tilting direction. For consistency in our later description, we mention the "right" or "left" direction of the radial fibers referencing to the elongation of the radial fiber, which then brings the "rightward tilting" toward the ACW rotation of the chiral pattern. To maintain the word "rightward tilting", we added the description to ensure accurate communication in our writing. We also rearrange the image in the new Figure 4A and Video 2 for better observation. Revision is made on page 8, lines 262 to 263.

(5) Why are the cells Figure 4A dominated by radial (and more-central, tilting fibers, while control cells in 4D show robust circumferential transverse arcs? Have these cells been plated for different amounts of time or is a different optical section shown?

We appreciate the reviewer's comment. The cells in Figure 4A and Figure 4D are prepared with similar conditions, such as incubation time and optical setting. Actin organization is a dynamic process, and cells can exhibit varied actin arrangements, transitioning between different forms such as circular, radial, chordal, chiral, or linear patterns, as they spread on a circular island (Tee et al., 2015). In Figure 4A, the actin is arranged in a chiral pattern, whereas in Figure 4D, the actin exhibits a radial pattern. These variations reflect the natural dynamics of actin organization within cells during the imaging process.

(6) All single-color images (such as Fig 5 F-actin) need to be black-on-white, since it is far more difficult to see F-actin morphology with red on black.

We appreciate the reviewer's comment. We have changed all F-actin images (single color) into black and white for better image clarity. Revisions are made in the new Figure 5, Figure 6 and Figure 7.

(7) Figure 5A, especially the F-actin staining, is quite a bit blurrier than other micrographs. These images should be replaced with images of comparable quality to those shown throughout.

We appreciate the reviewer's comment. We agree that the F-actin staining in Figure 5 is difficult to observe. To improve image clarity, the F-actin staining images are replaced with more zoomed-in image. Revision is made in the new Figure 5.

(8) F-actin does not look unchanged by Y27632 treatment, as the authors state in line 306. This may be partially due to image quality and the ambiguities of communicating with the blue-to-red colormap. Similarly, I don't agree that mDia2 depletion did not change F-actin distribution (line 330) as cells in that condition had a prominent peripheral ring of F-actin missing from cells in other conditions.

We appreciate the reviewer's comment. We agree with the reviewer's observation that the F-actin distribution is indeed changed under Y27632 treatment compared to the control in Figure 5A-B. Here, we would like to emphasize that the actin ring persists despite the actin structure being altered under the Y27632 treatment. The actin ring refers to the darker red circle in the distribution heatmap. It presents the condensed actin structure, including radial fibers and transverse arcs. This important structure remains unaffected despite the disruption of myosin II, the key component in radial fiber.

Furthermore, we agree with the reviewer that mDia2 depletion does change F-actin distribution. Similar to the Y27632 treatment, the actin ring persists despite the actin structure being altered under mDia2 gene silencing. Moreover, compared to other treatments, mDia2 depletion has less significant impact on actin distribution. To address these points more comprehensively, we have made revision in Y27632 treatment and mDia2 sections. The revisions of Y27632 and mDia2 are made on pages 10, lines 324-327 and 352-353, respectively.

(9) The colormap shown for intensity coding should be reconsidered, as dark red is harder to see than the yellow that is sub-maximal. Verdis is a colormap ranging from cooler and darker blue, through green, to warmer and lighter yellow as the maximum. Other options likely exist as well.

We appreciate the reviewer's comment. We carefully considered the reviewer's concern and explored other color scale choices in the colormap function in Matlab. After evaluating different options, including "Verdis" color scale, we found that "jet" provides a wide range of colors, allowing the effective visual presentation of intensity variation in our data. The use of 'jet' allows us to appropriately visualize the actin ring distribution, which represented in red or dark red. While we understand that dark red could be harder to see than the sub-maximal yellow, we believe that "jet" serves our purpose of presenting the intensity information.

(10) For Figure 6, why doesn't average distribution of NMMIIa look like the example with high at periphery, low inside periphery, moderate throughout lamella, low perinuclear, and high central?

We appreciate the reviewer's comment. We understand that the reviewer's concern about the average distribution of NMMIIa not appearing as the same as the example. The chosen image is the best representation of the NMMIIa disruption from the transverse arcs after the mDia2

silencing. Additionally, it is important to note that the average distribution result is a stacked image which includes other images. As such, the NMMIIA example and the distribution heatmap might not necessarily appear identical.

(11) In 2015, Tee, Bershadsky and colleagues demonstrated that transverse bundles are dorsal to radial bundles, using correlative light and electron microscopy. While it is important for Kwong and colleagues to show that this is true in their cells, they should reference Tee et al. in the rationale section of text pertaining to Figure 7.

We appreciate the reviewer's comment. Tee, et al (Tee et al., 2015) demonstrated the transverse fiber is at the same height as the radial fiber based on the correlative light and electron microscopy. Here, using the position of myosin IIa, a transverse arc component, our results show the dorsal positioning of transverse arcs with connection to the extension of radial fibers (Figure 7C), which is consistent with their findings. It is included in our manuscript, page 12, lines 421 to 424, and page 14 lines 477 to 480.

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