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Report

Anillin Is a Scaffold Protein That Links RhoA, Actin, and Myosin during Cytokinesis

Alisa J. Piekny[1] and Michael Glotzer[1,*]
[1]Department of Molecular Genetics and Cell Biology
University of Chicago
Chicago, Illinois 60637

Summary

Cell division after mitosis is mediated by ingression of an actomyosin-based contractile ring. The active, GTP-bound form of the small GTPase RhoA is a key regulator of contractile-ring formation. RhoA concentrates at the equatorial cell cortex at the site of the nascent cleavage furrow. During cytokinesis, RhoA is activated by its RhoGEF, ECT2. Once activated, RhoA promotes nucleation, elongation, and sliding of actin filaments through the coordinated activation of both formin proteins and myosin II motors (reviewed in [1, 2]). Anillin is a 124 kDa protein that is highly concentrated in the cleavage furrow in numerous animal cells in a pattern that resembles that of RhoA [3–7]. Although anillin contains conserved N-terminal actin and myosin binding domains and a PH domain at the C terminus, its mechanism of action during cytokinesis remains unclear. Here, we show that human anillin contains a conserved C-terminal domain that is essential for its function and localization. This domain shares homology with the RhoA binding protein Rhotekin and directly interacts with RhoA. Further, anillin is required to maintain active myosin in the equatorial plane during cytokinesis, suggesting it functions as a scaffold protein to link RhoA with the ring components actin and myosin. Although furrows can form and initiate ingression in the absence of anillin, furrows cannot form in anillin-depleted cells in which the central spindle is also disrupted, revealing that anillin can also act at an early stage of cytokinesis.

Results and Discussion

Active RhoA and Anillin Modulate Each Other's Localization

In dividing HeLa cells, anillin concentrates at the equatorial cortex upon anaphase onset, where it colocalizes extensively with RhoA, a key regulator of furrow formation and ingression (Movie S1 available online, Figure 1A). In human cells, RhoA accumulation at the equatorial cortex can be observed after fixation with trichloroacetic acid (TCA) [8]. Fluorescently tagged *C. elegans* RhoA becomes highly concentrated on the equatorial cortex during anaphase in living cells, providing independent demonstration of a physiologically relevant cortical pool of RhoA [9]. Anillin-depleted cells initiate furrowing but undergo cortical oscillations and then furrow regression, suggesting that anillin promotes furrow stabilization [6, 7]. Although furrows ingress extensively, anillin depletion unexpectedly causes a dramatic decrease in the TCA-fixable cortical pool of RhoA (17/17 cells; Figure 1B) [7]. Furthermore, prevention of oscillations with the nonmuscle myosin inhibitor blebbistatin does not restore RhoA localization in anillin-depleted cells (31/31 cells; Figure 1B). However, live imaging reveals that GFP:RhoA (GFP: green fluorescent protein) localizes to the equatorial cortex in anillin-depleted cells (Movie S2). Conversely, anillin localization requires RhoA. RhoA-depleted cells contain trace levels of anillin at the equatorial region (17/17 cells) (Figure 1C). Anillin localization is also lost in cells depleted of the RhoA exchange factor ECT2 (23/23 cells; Figure 1D) [7]. Collectively, these data indicate that active RhoA is essential for anillin localization and that anillin stabilizes RhoA for fixation.

The Anillin Homology Domain Is Essential for Function and Localization

Anillin contains actin and myosin binding domains, an anillin homology domain (AHD), named for its conservation among metazoan anillins (Figure S1), and a pleckstrin homology (PH) domain [3, 5, 6]. To study the functions of these domains, we developed an RNA interference (RNAi) knockdown and transfection rescue assay in human cells. After 24 hr of small interfering RNA (siRNA) treatment, anillin-depleted cells show a penetrant phenotype (8/9 cells observed by live imaging undergo oscillations and fail to divide) that is rescued by coexpression of RNAi-resistant GFP:anillin (13/13 cells were rescued). We conducted a functional domain analysis of anillin by assessing the fraction of binucleated, transfected cells ($n > 300$ cells per construct, Figure 2A). In an asynchronous population of cells fixed after 24 hr of RNAi treatment, 40%–50% of cells are binucleate. Cotransfection of full-length anillin substantially rescues the percentage of binucleate cells and localizes like endogenous anillin (construct A, Figures 2A and 2B). The nuclear localization signal (NLS) sequences are not required for cytokinesis, nor are they required for furrow localization (construct B; Figures 2A and 2B). However, the C terminal PH domain is required for robust furrow localization and for anillin function (construct H). Constructs lacking either the myosin (construct C) or actin (construct D) domains are partially functional, but constructs lacking both (constructs E and F) are nonfunctional. However, these constructs localize discretely to an equatorial region that shifts minimally as the cell oscillates (Figures 2A and 2B, Movie S1). A construct lacking the conserved AHD (construct J) fails to rescue anillin depletion. The AHD is required for persistent equatorial localization during furrowing because construct J becomes localized in polar regions of the cell as it oscillates instead of remaining equatorial like constructs E and F (Figures 2A and 2B, Movie S1).

*Correspondence: mglotzer@uchicago.edu

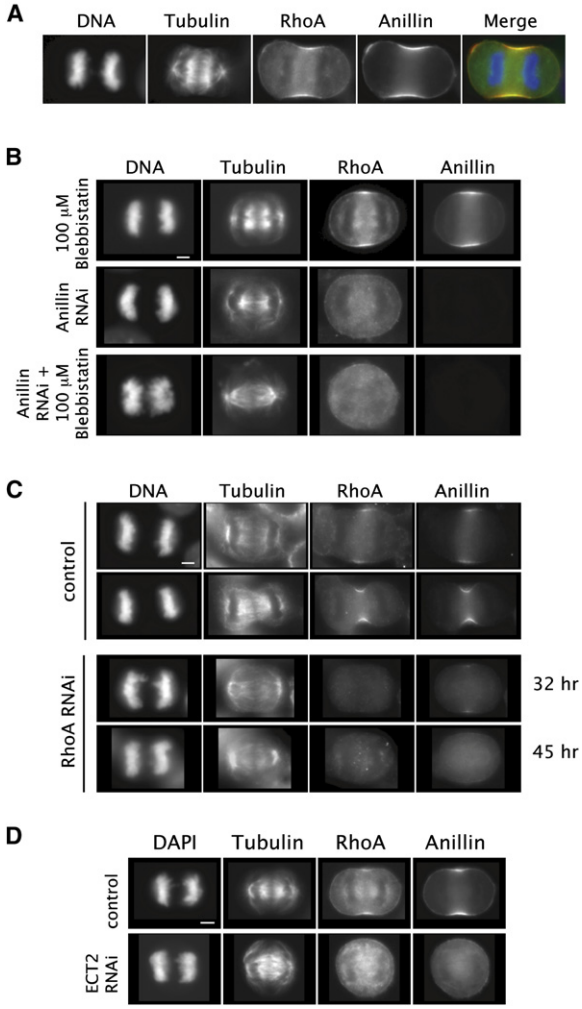

Figure 1. Anillin Localization to the Cleavage Furrow Requires Active RhoA, and Anillin Stabilizes RhoA to Fixation

(A) A HeLa cell fixed with TCA and costained for DNA (blue), Tubulin, RhoA (green), and anillin (red).

(B) HeLa cells treated with Blebbistatin (100 μM), anillin RNAi, or both were fixed with TCA and costained for DNA, Tubulin, RhoA, and anillin.

(C) HeLa cells were treated with control RNAi or RhoA RNAi for 32 or 45 hr, then fixed with TCA and costained for DNA, Tubulin, RhoA, and anillin.

(D) HeLa cells were treated with ECT2-specific siRNAs or with reagent alone, then fixed with TCA and stained for DNA, Tubulin, RhoA, and anillin (top panels).

Scale bar for all mitotic cells represents 5 μm.

Construct I, which contains just the AHD, displays weak furrow localization, supporting its role in anillin localization (Figures 2A and 2B, Movie S1). All constructs were assayed for their ability to restore RhoA localization in TCA-fixed cells. Constructs A–G concentrate at the furrow and fully stabilize RhoA for fixation. Constructs H and I weakly accumulate at the furrow but nevertheless allow detection of RhoA. Notably, construct J, which lacks the AHD, fails to stabilize RhoA even though it initially concentrates at the furrow (Figure 2C). Thus, the AHD mediates persistent furrow localization and stabilization of RhoA.

Anillin Shares Homology with Rhotekin and Binds to RhoA

Profile-based searching revealed that the AHD is structurally related to Rhotekin (Figure 3A and Figure S1) [10]. The region of similarity extends from Rhotekin's RhoA-GTP binding domain to its PH domain (Figure 3A). The AHD also has a short motif of high homology to the Rho binding region of Protein Kinase N (PKN). Because Rhotekin and PKN selectively bind to active RhoA [11, 12], we determined whether anillin could bind to RhoA. To assess RhoA binding, we expressed GFP:RhoA by transient transfection. GFP:RhoA migrates on sodium dodecyl sulfate (SDS) gels as two distinct species. The AHD (residues 608–943) binds to the faster migrating band of the doublet and less avidly to the slower migrating species. The efficiency of binding to the faster migrating species is enhanced by the RhoA-activating mutation Q63L (Figure 3B). The slower migrating species appears to be a modified form of RhoA. Overexpression of untagged RhoA also results in a fragment that migrates more slowly than endogenous RhoA, and a small fraction of endogenous RhoA also runs with this reduced mobility (Figure S2A). Furthermore, cleavage of GFP:RhoA at a protease site engineered between GFP and RhoA releases a species of RhoA that migrates more slowly than endogenous RhoA (Figure S2B). The RhoA binding domain (RBD) from Rhotekin also preferentially binds the faster migrating form (Figure S2C). To further address anillin's nucleotide specificity, we performed pull-down experiments after preloading GFP:hsRhoA in lysates with either guanine diphosphate (GDP) or guanine triphosphate (GTP) (Figure 3C). GFP:RhoA-loaded with GTP enhanced anillin binding as compared to GDP. To determine whether anillin can directly bind to RhoA, we performed in vitro assays with recombinant proteins. Indeed, hsRhoA-GTP directly bound to anillin, although with lower affinity than the RBDs from Rhotekin or mDia1 (Figure 3D, Figure S2D).

To further analyze the relevance of the Rho binding region for anillin function, we mutated the conserved residues 837 DFEINIE 843 to AFAINIA. Full-length anillin with these substitutions (Figure 2A, construct L) fails to rescue the anillin phenotype, and neither the full-length nor a shorter construct with these mutations (Figure 2A, construct K) can efficiently stabilize RhoA during TCA fixation (Figure 3E). These mutations also reduce the efficiency with which the C terminus of anillin (608–1087) pulls down GFP:RhoA (Figure 3B). Mutation of another set of residues (800 KAGAEN 805 to AAGAAA), which are conserved in PKN and are part of a RhoA binding interface [12], had no effect on anillin function or localization (data not shown), confirming the specificity of the DFEINIE region. Unfortunately, the interaction of RhoA with Rhotekin has not been analyzed at the atomic level, precluding precise mutational analysis. Furthermore, many RhoA effectors have several RhoA binding sites, which might act cooperatively [13]. Additional evidence for an in vivo interaction between anillin and RhoA is the finding that construct F, consisting of the AHD and PH domains, induces the formation of ectopic RhoA-containing foci in interphase cells (41% of cells, n = 151; Figure S3). These foci are less prominent in cells expressing an equivalent construct (K) with the alanine

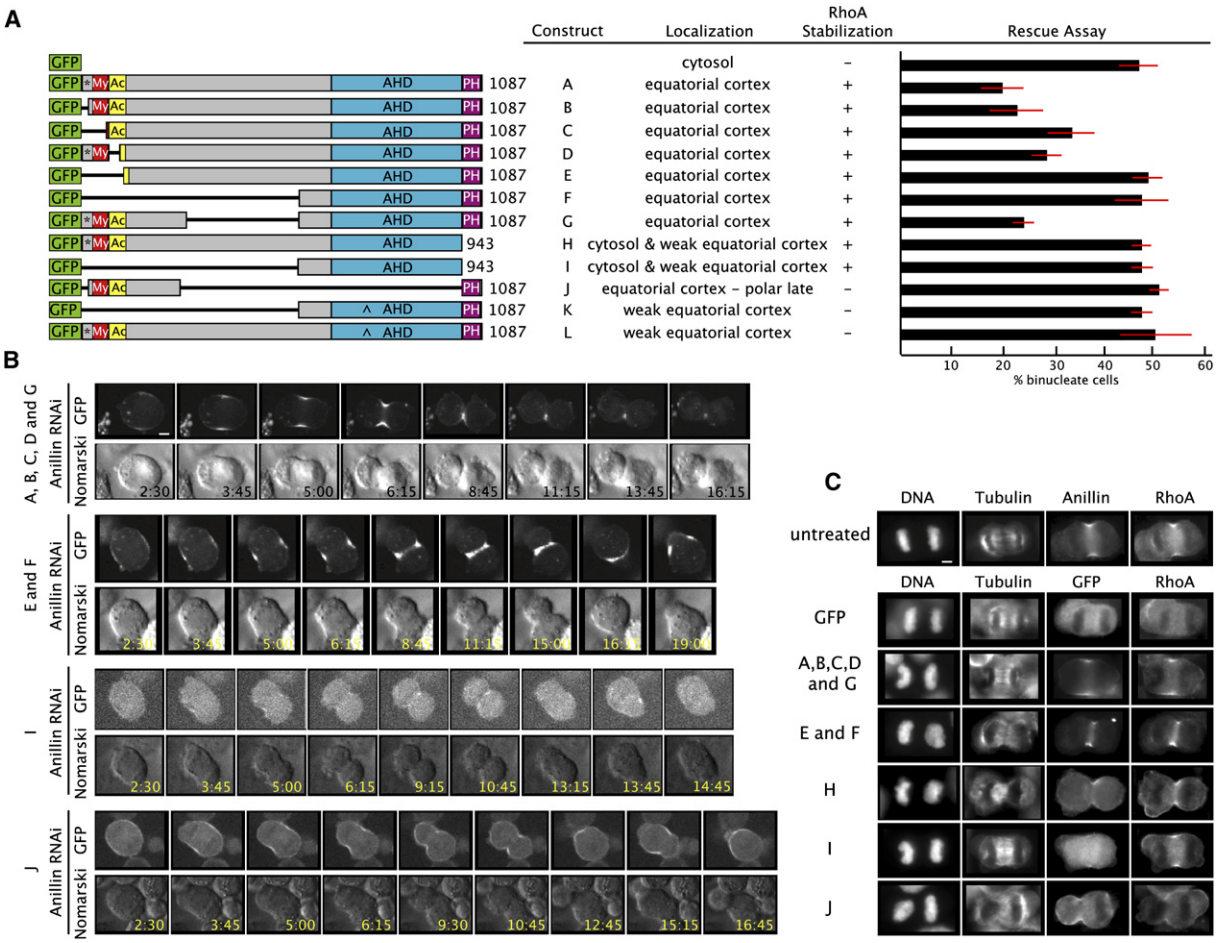

Figure 2. The Anillin Homology Domain Contributes to the Function and Equatorial Localization of Anillin

(A) A schematic outlining the constructs used for localization in live cells, RhoA localization in TCA fixed cells, and results of a functional rescue assay. Rescue assays were scored as the percentage of GFP-positive cells that were binucleate (>300 cells), and error bars indicate standard deviations. Conserved regions and previously described domains are indicated: "My" is the myosin binding domain (aa 146–258), "Ac" is the actin binding and bundling region (aa 258–371), "AHD" is the anillin homology domain (aa 608–943), and "PH" is the pleckstrin homology domain (aa 943–1087). * indicates 2× NLS sequences, and ^ refers to the mutation 837 DFEINIE 843 – AFAINIA.

(B) HeLa cells expressing the indicated RNAi-resistant GFP:anillin constructs, cotransfected with anillin siRNAs. Selected time points were taken from time-lapse recordings of live cells. Times are from anaphase onset. Constructs that exhibited similar localization are indicated.

(C) Cells expressing the indicated RNAi-resistant GFP:anillin constructs, cotransfected with anillin siRNAs, were fixed with TCA and costained for DNA, Tubulin, GFP, and RhoA. Constructs with similar rescue, localization, and RhoA localization are indicated.

substitutions described above (6% of cells, n = 113; Figure S3). Furthermore, these foci are ECT2 independent, unlike AHD localization during anaphase. Previous overexpression studies had also suggested that RhoA and anillin interact [14]. Collectively, these results indicate that anillin is structurally related to Rhotekin and can bind to RhoA, and we have mapped a domain critical for anillin localization and function.

Anillin Is Required to Maintain Myosin in the Contractile Ring

To further investigate anillin's cytokinetic role, we compared the localization of RhoA and myosin in the presence and absence of anillin. In control cells, RhoA, anillin, and myosin colocalize throughout cytokinesis. In anillin-depleted cells, live-cell imaging reveals furrow accumulation of GFP:RhoA (Figure 4A). This accumulation of GFP:RhoA is consistent with the contractile behavior of anillin-depleted cells and suggests that anillin stabilizes RhoA during TCA fixation. When these cells undergo oscillations, GFP:RhoA remains localized in the equatorial plane (Figure 4A). The cytoplasmic contents move through this plane, and although RhoA shifts somewhat, it remains discretely localized. However, myosin II exhibits entirely different dynamics, as visualized with GFP:mlc or the phosphomimetic form GFP:mlcE20E21. Myosin is initially equatorial, but after furrow ingression begins, myosin localizes to the polar regions of the cell, moving in concert with the oscillations (Figure 4B). Inhibition of myosin activity with blebbistatin results in the immediate cessation of oscillations (data not shown). These results suggest that anillin promotes the anchoring of myosin in the vicinity of RhoA. Immunolocalization of myosin in cells expressing various anillin constructs substantiate this conclusion. Although anillin constructs E and F colocalize with

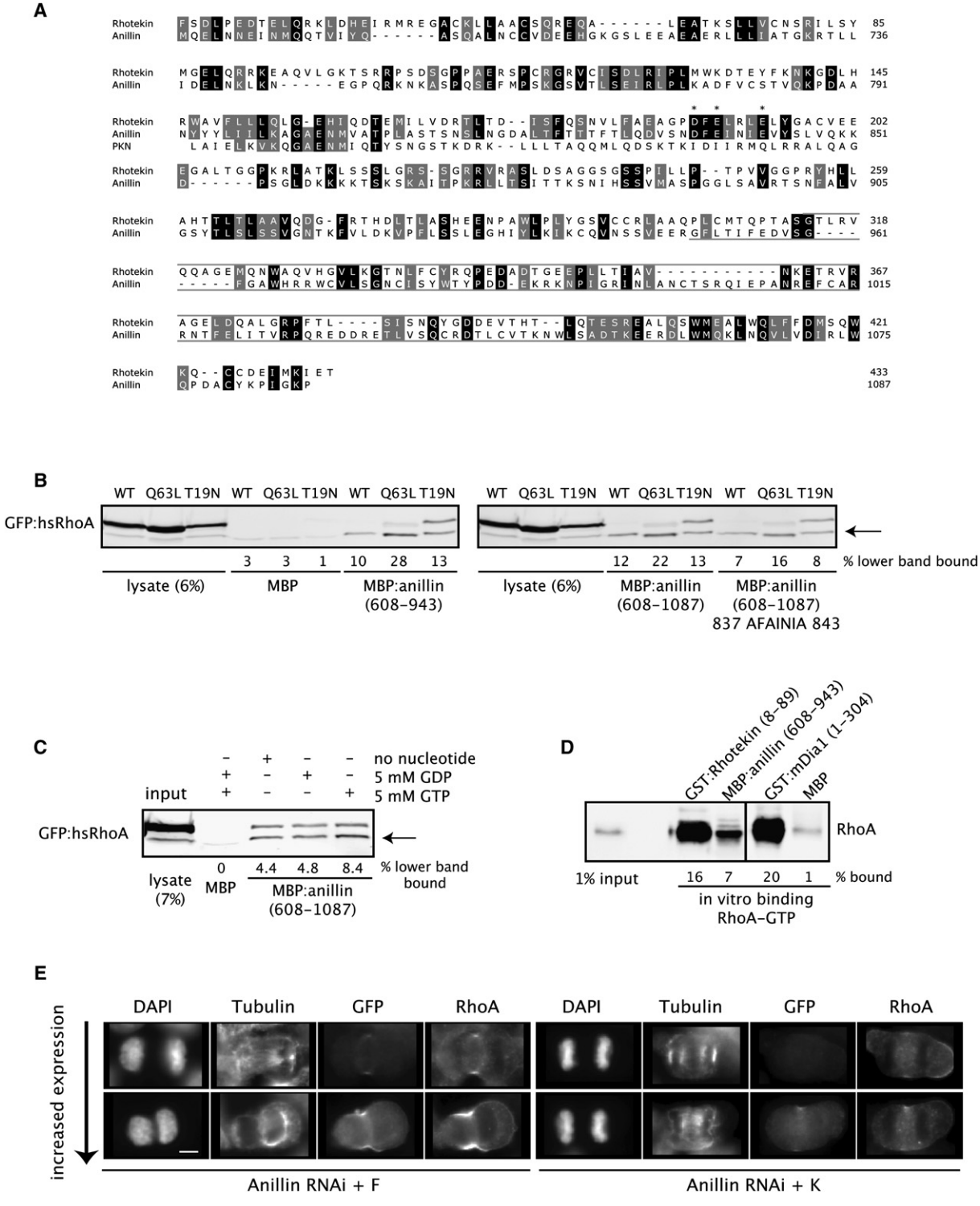

Figure 3. The Anillin Homology Domain Binds to RhoA

(A) An alignment of the conserved AHD region with the RhoA binding protein Rhotekin and a region of PKN (accession numbers AAH70066.1, NP_001015055.1, and BAA05169). The PH domains are indicated by the gray line, and the 837 DFEINIE 843 – AFAINIA mutations are also indicated by asterisks. Identical amino acids are shaded in black, and conserved residues are in gray.

(B) Lysates from HEK293 cells, transfected with GFP:hsRhoA, GFP:hsRhoAQ63L, and GFP:hsRhoAT19N, were used in pull-down assays with MBP, MBP:anillin (aa 608–943), MBP:anillin (aa 608–1087), and MBP:anillin (aa 608–1087; 837 DFEINIE 843 – AFAINIA).

(C) Lysates from HEK293 cells transfected with GFP:hsRhoA, loaded with 5 mM GDP or GTP, were used in pull-down assays with MBP and MBP:anillin (aa 608–1087).

(D) Western blot of in vitro binding with GTP-loaded RhoA as input with the indicated proteins on beads and probed for hsRhoA.

(E) HeLa cells transfected with anillin siRNAs and either construct F or K were costained for DNA, Tubulin, GFP, and RhoA. Cells of similar levels of expression were chosen for direct comparison.

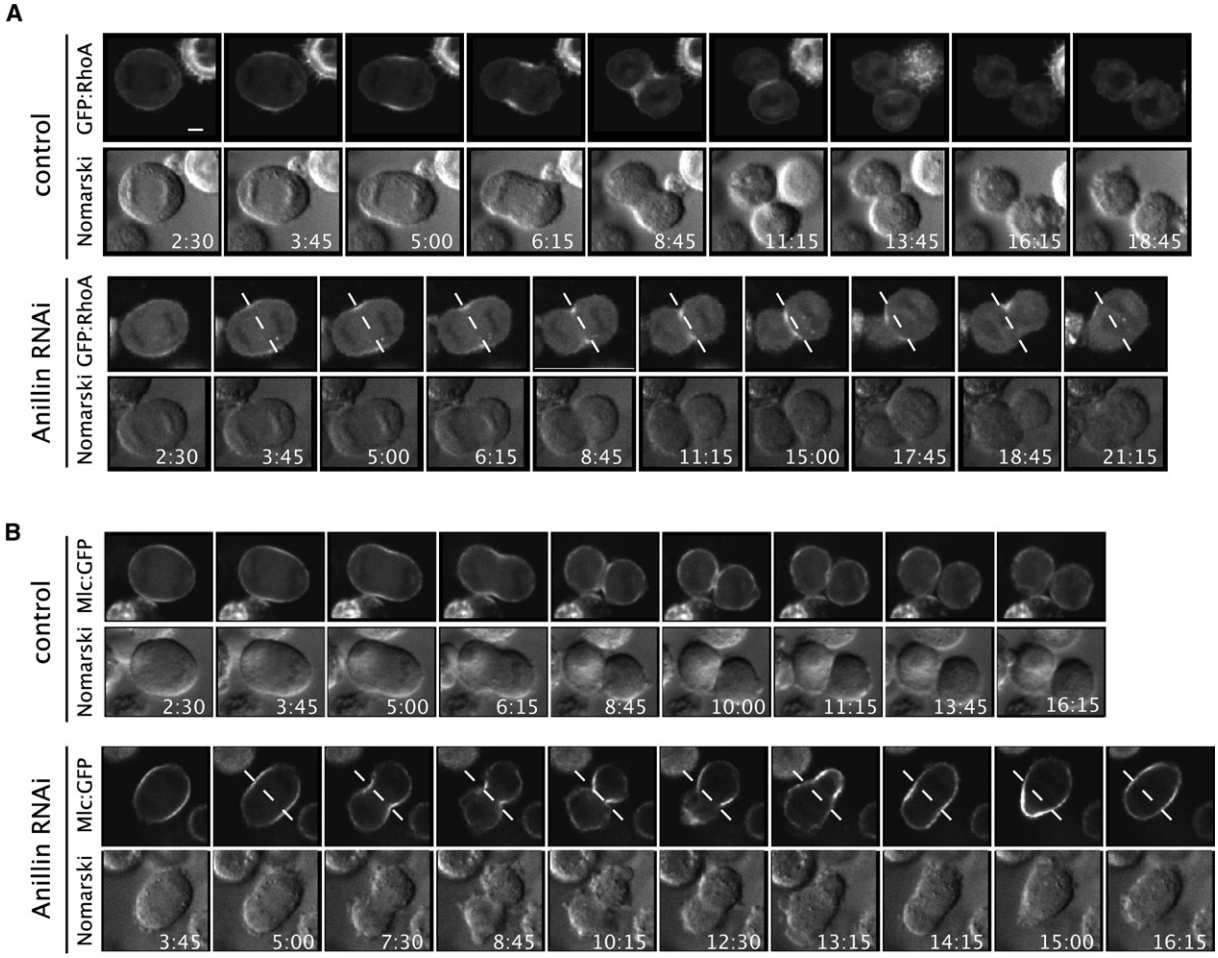

Figure 4. Anillin Is Required to Retain Myosin at the Equator

Time-lapse imaging of control or anillin-depleted HeLa cells expressing GFP:ceRhoA (A) or GFP:mlc (B) is shown. Times are indicated from anaphase onset. The dashed line marks the equatorial plane of the dividing cells.

myosin in the equatorial plane, they do not colocalize when myosin concentrates in the polar regions (Figure S4). Dual color time-lapse imaging reinforces this conclusion. Construct F (containing the AHD domain) and RhoA colocalize throughout furrowing. In contrast, construct F and myosin light chain colocalize equatorially, but not when myosin becomes enriched in the polar regions (Movie S2). In contrast, construct J, which lacks the AHD, colocalizes with myosin in the polar regions (Figure S4). Thus, anillin acts as a cytokinetic scaffold to link RhoA with the contractile-ring components actin and myosin. Depletion of anillin allows myosin to escape from the equatorial plane, where RhoA remains concentrated. Myosin accumulates in polar regions of the cell, where it causes a contraction and induces expansion at the opposite pole. This mechanical stress might suffice to induce myosin recruitment at the expanded pole, establishing cortical oscillations [15–17].

Anillin Is Required for Furrow Formation in the Absence of a Central Spindle

Although anillin directly binds to three critical contractile-ring components and is among the first proteins to localize to the furrow upon anaphase onset, cytokinesis can progress to a remarkable extent or even complete in its absence [4–7]. Because parallel pathways regulate furrow formation in animal cells [18, 19], an early function for anillin could be obscured by functional redundancy. Therefore, we determined whether anillin contributes to furrow formation in cells in which cytokinesis is partially perturbed. Depletion of the kinesin component of centralspindlin, MKLP1, causes disruption of the central spindle, leading to mislocalization of ECT2 and expansion of the equatorial zone containing contractile-ring components [9]. However, half of these cells furrow. Recent evidence suggests that astral microtubules locally modulate contractility by inhibiting recruitment of contractile-ring components [20]. Although depletion of either anillin or MKLP1 affects RhoA localization, in neither case does RhoA enter the most polar regions of the cell. In cells depleted of both MKLP1 and anillin, RhoA expands around the entire cortex of the cell, and furrows uniformly fail to form (Movie S3). Further, myosin and actin also expand around the entire cortex (27/27 cells for actin and 23/23 cells for myosin, Figure 5). Thus, the central spindle and anillin can independently promote RhoA localization and furrow formation. In the absence of the central spindle, anillin restricts myosin localization and thereby induces contractile-ring assembly.

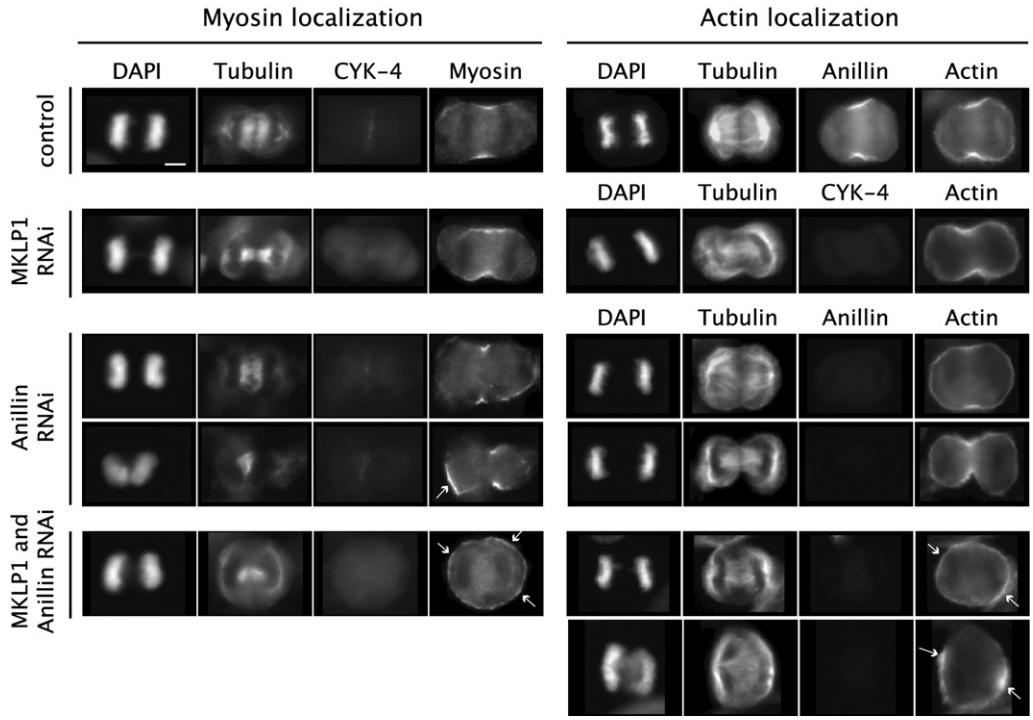

Figure 5. Anillin Is Essential for Furrow Formation in Cells Lacking a Central Spindle

HeLa cells treated with reagent alone or siRNAs specific for MKLP1, anillin, or both were fixed with either MeOH or 4% paraformaldehyde (PFA). MeOH-fixed cells were stained for DNA, Tubulin, CYK-4, and myosin (left panels), and PFA-fixed cells were stained for DNA, Tubulin, anillin, and actin (right panels). Localization outside the furrow is marked with arrows.

Conclusions

In both *S. pombe* and animal cells, anillin-like proteins are early markers of the nascent cleavage furrow [3, 21, 22]. In most systems, anillin is not required for furrow formation. *C. elegans* have three anillin-like proteins, and *ani-1* is required for contractile events in the early embryo, such as membrane ruffling and pseudocleavage, but is not strictly required for cytokinesis [4]. *Drosophila* anillin is required for cellularization in early embryos and promotes late cytokinetic events in S2 cells [23, 24]. Fission yeast express two anillin-related proteins, Mid1p and Mid2p, which regulate proper positioning of the division plane and septation, respectively [21, 25–27]. Whereas in human cells anillin localizes via its C-terminal Rho binding domain, in *S. pombe* mid1p localization involves a C-terminal amphipathic helix [28]. Interestingly, the AHD is not well conserved in mid1p, nor is there compelling evidence that RhoA triggers contractile-ring formation in *S. pombe*. Thus, various organisms might employ distinct mechanisms to recruit anillin, which executes its conserved function as a scaffold protein in the contractile ring.

Supplemental Data

Experimental Procedures, four figures, and three movies are available at http://www.current-biology.com/cgi/content/full/18/1/30/DC1/.

Acknowledgments

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