

The Myriad Roles of Anillin during Cytokinesis

Alisa J. Piekny¹ and Amy Shaub Maddox²

1. Department of Biology, Concordia University, 7141 Sherbrooke St. W, Montréal, QC, Canada H4B 1R6 2. Institute for Research in Immunology and Cancer, Department of Pathology and Cell Biology, P.O. Box 6128, Station Centre-Ville, Montréal, QC, Canada H3C 3J7

Corresponding authors: AJP: apiekn@alcor.concordia.ca, ASM: amy.maddox@umontreal.ca

Abstract: Anillin is a highly conserved multidomain protein that interacts with cytoskeletal components as well as their regulators. Throughout phylogeny, Anillins contribute to cytokinesis, the cell shape change that occurs at the end of meiosis and mitosis to separate a cell into daughter cells. Failed cytokinesis results in binucleation, which can lead to genomic instability. Study of Anillin in several model organisms has provided us with insight into how the cytoskeleton is coordinated to ensure that cytokinesis occurs with high fidelity. Here we review Anillin's interacting partners and the relevance of these interactions *in vivo*. We also discuss questions of how these interactions are coordinated, and finally provide some perspective regarding Anillin's role in cancer.

Keywords: cytoskeleton; cytokinesis; Rho; F-actin; cellularization

Abbreviations: F-actin (filamentous actin), GEF (guanine nucleotide exchange factor), GAP (GTPase activating protein), PH (pleckstrin homology), AH (Anillin homology), SH3 (Src homology 3), TCA (trichloroacetic acid), FRET (fluorescence/Förster resonance energy transfer), NLS (nuclear localization signal), APC (anaphase promoting complex), D-box (destruction box), SIN (septation initiation network), RNAi (RNA-mediated interference)

1. Introduction

Cell shape is dictated in large part by the actomyosin (F-actin and non-muscle myosin II) cytoskeleton, and its myriad associations with the plasma membrane. Many temporal and spatial cues that govern the dynamics of actin, myosin and thus cell shape come from the microtubule cytoskeleton. Cytokinesis, a particularly dramatic cell shape change, is the physical division of one cell into two daughter cells. During cytokinesis, the cell faces several challenges. First, the cell equator must be specified by microtubules of the anaphase spindle, so that cytokinesis is spatially and temporally coordinated with chromosome segregation (reviewed in [1, 2]). Second, the small GTPase Rho must be locally activated by the GEF Ect2 and GAP Cyk-4 [3]. Rho activity elicits the assembly and constriction of a contractile ring rich in actin filaments and active myosin. This ring must function efficiently to narrow the cytoplasmic connection between the nascent daughter cells. Last, the daughter cells must permanently separate at the cell-cell bridge (midbody). Anillin is a conserved multidomain protein, which, due its many interactions, is a prime candidate for scaffolding and organizing the cytoskeleton and its regulators in all the above events (also recently reviewed in [4-6]).

2. Roles of Anillin as revealed by binding partners

2.1 Actin

Anillin was first isolated from *Drosophila* embryo extracts by virtue of its binding to phalloidin-stabilized F-actin (Table 1). This association was specific for F-actin, since Anillin was not enriched among proteins bound to a G-actin column [7]. The antibody raised against "Antigen 8" (Anillin) recognized contractile structures including cellularization furrows (Fig. 1) in *Drosophila* stage 14 embryos and cytokinetic rings later in development. *Drosophila* Anillin was cloned and found to be encoded by the *scraps* gene [8].

Anillin is also capable of bundling actin filaments. While amino acids 258-340 of *Drosophila* Anillin are sufficient for F-actin binding, amino acids 246-371 bundle actin filaments. A small fragment with strong (salt-resistant) F-actin bundling activity was found to be monomeric by analytical ultracentrifugation, suggesting this single domain has two F-actin binding sites. However, this fragment could dimerize after binding F-actin [9]. Anillin's ability to bind and bundle F-actin is conserved, as *Xenopus* Anillin also robustly bundles F-actin (amino acids 245-418; [10]).

F-actin and Anillin are recruited independently to the contractile ring in human and *Drosophila* cultured cells, as well as to meiotic rings in *Drosophila* spermatogenesis [11-14]. However, F-actin increases the efficiency of Anillin targeting to the cell equator in both systems (in terms of timing or spatial accuracy) [13-15].

Importantly, Anillin does not bind all F-actin in the cell. Rather, it is specific to cell division-related contractile structures (Fig. 2) and, for example, is absent from the apically constricting cells during *Drosophila* dorsal closure [9] and stress fibers in interphase mammalian cultured cells ([10] and A.S. Maddox, unpublished observations). Anillin has been widely implicated in "organizing contractility" (see 2.2-2.3, 2.5-2.6), but its direct effects on F-actin organization are poorly understood. F-actin was assumed to become delocalized as Anillin depleted cells undergo lateral furrow oscillations (see 2.2), but it was only recently shown that Anillin depletion allows F-actin, like myosin, outside the equator in *Drosophila* spermatocytes that fail cytokinesis [12]. It is not known whether Anillin regulates the formation of parallel or antiparallel actin bundles in cells. By regulating actin bundling, Anillin may increase the efficiency of actomyosin contractility.

Anillin also interacts indirectly with F-actin via binding to the formin mDia2 (amino acids 1-91 of human Anillin and 156-533 of mDia2, which includes the DID region) possibly to stabilize formin in the active conformation after it binds RhoA [16]. The formin-Anillin interaction is important for the cortical localization of mDia2 and for the success of cytokinesis [16]. This interaction likely explains why depletion of mDia2 causes the ectopic localization of RhoA, F-actin, myosin and Anillin and results in cell oscillations similar to those that occur following loss of Anillin [17]. Thus, Anillin may not only organize actin, but also promote its polymerization in the contractile ring.

2.2 Myosin

While Anillin interacts indirectly with myosin via F-actin, there is also a direct interaction between Anillin and non-muscle myosin II (Table 1). Approximately 100 amino acids near the N-terminus (residues 143-245) of *Xenopus* Anillin are necessary and sufficient for binding myosin. This interaction is independent of F-actin (it was assessed in the presence of the actin-depolymerizing drug Cytochalasin D) and requires an activating phosphorylation of myosin regulatory light chain [18].

In vertebrates, *Drosophila* and *C. elegans*, Anillin (ANI-1 in *C. elegans*) and myosin are recruited to the contractile ring independently [13, 18, 19]. The only clear exception to this rule is in the fission yeast *S. pombe*, wherein the Anillin-like protein Mid1 is required for the equatorial recruitment of myosin, and all other contractile ring proteins.

If the interaction between Anillin and myosin does not serve the purpose of recruitment in metazoan cells, what is its role? One of Anillin's key functions is to "organize" myosin. *Drosophila* Anillin is required for the organization of myosin into discrete, intact rings throughout the cellularization front ([8]; Fig. 1). Depletion of *Drosophila* or human Anillin from cultured cells perturbs the temporal and spatial stability of myosin at the cell equator during cytokinesis [12, 13, 18, 20-22]. *C. elegans* Anillin (ANI-1) organizes myosin into dynamic foci during polarity establishment and cytokinesis [19, 23] and promotes asymmetric furrow ingression in the zygote (Fig. 3; [24]). *C. elegans* ANI-2 is required for the integrity of the myosin-rich, potentially contractile lining of the *C. elegans* oogenic gonad [19]. Specifically, Anillin may increase the effective processivity of non-muscle myosin II, which is known to have extremely low intrinsic processivity, by coordinating myosin with bundled F-actin and increasing its chance of encountering an actin track [25].

Neither of the predicted [15, 18] myosin- or actin-binding domains of human Anillin is required for its localization to the equatorial cortex [21]. A truncation lacking both of these domains localizes properly, but fails to replace the function of depleted endogenous Anillin. Specifically, cytokinesis failure results from a lateral oscillation behavior during which the polar regions of the cell become aberrantly contractile and push cytoplasm across the equator (Fig. 4; [21]). This contractile behavior in the polar regions of the cell likely is caused by the mislocalization of myosin outside the cell equator during these oscillations (Fig. 4). Interestingly, while this abnormal oscillation occurs in cells expressing Anillin that cannot bind actin or myosin, the truncated protein itself does not leave the equator to follow myosin to the poles. This suggests that C-terminal domains of Anillin (see 2.3, 2.5-2.8) are sufficient for equatorial targeting, and that the N-terminal domains organize contractility (Fig. 2; see 2.2).

2.3 Septins

Anillin also interacts with the septins (Table 1), which comprise a conserved family of GTP-binding proteins that heterooligomerize into nonpolar rods and further associate to generate filaments and sheets [26]. Like Anillin, septins are enriched in contractile structures in many cellular contexts [27]. Specifically during cytokinesis and cellularization, this localization depends on association with Anillin (Fig. 2).

Direct interaction between septins and Anillin was demonstrated using a minimal reconstituted heterooligomer of human septins 2, 6 and 7 that bound *Xenopus* Anillin *in vitro* [10]. Septin binding activity is conferred by the C-terminal third of Anillin, which encompasses the terminal PH domain (Fig. 5) and the upstream sequence known as the "Anillin Homology Domain" named due to phylogenetic conservation [15, 19]. A smaller region (the PH domain plus the adjacent N-terminal 46 amino acids) is sufficient for association with ectopic septin-containing structures in mammalian cells [15].

In *Drosophila* embryos expressing Anillin variants with mutations in the predicted septin-binding domain, and cultured cells and spermatocytes depleted of Anillin, the maintenance and organization of septins (Peanut and Sept2) on cellularization furrows (Fig. 1) and contractile rings is perturbed [8, 12, 13]. The reciprocal relationship is also true: Peanut is required to maintain and organize Anillin on cellularization furrows [28]. In human cells depleted of Anillin, an Anillin truncation lacking the AH domain, which may mediate interaction with septins, mislocalizes to the poles of the cell during oscillations, similar to myosin [21]. An

Anillin truncation lacking the PH domain, another region involved in septin interactions, cannot localize to the cortex and elicits defects even in the presence of endogenous Anillin [21]. Conversely, *C. elegans* Anillin (ANI-1) is required for septin enrichment in the contractile ring but septins are dispensable for quantitatively normal recruitment of Anillin to the contractile ring ([19]; A.S. Maddox, unpublished observations). However, *C. elegans* septins are required for Anillin's asymmetric localization during ring closure [24] suggesting that throughout phylogeny, septins and Anillin are interdependent for their recruitment and organization.

Indeed, the *S. pombe* Anillin-like protein Mid2 depends on septins for its equatorial localization [29]. Mid2 is not required for the recruitment of septins to the division plane, but does affect their dynamics; in *mid2* mutants, septins exchange abnormally quickly at the cell equator and localize aberrantly to the nascent cell-cell boundary [30]. These effects on septin dynamics help explain Mid2's importance for timely septation [29, 30].

Septins can directly interact with lipids [31, 32], and septin sheets have been observed in close apposition to the plasma membrane [33]. Thus, it is possible that septins, via Anillin, help link the actin cytoskeleton to the membrane. Since septins form repeating linear arrays, a septin-Anillin-F-actin or septin-Anillin-myosin linkage could generate higher-order organization of the cortical cytoskeleton.

2.4 CD2AP

Human and *Drosophila* Anillins interact with CD2AP (cluster of (leukocyte) differentiation 2-associated protein; Cindr in *Drosophila*; Table 1), adaptor proteins implicated in membrane trafficking and actin remodeling (see references within [34]). CD2AP localizes to the spindle midzone in anaphase and the center of the midbody in telophase in human cells; its first two SH3 domains are necessary and sufficient for this localization [34]. Cindr localizes to the contractile ring and transiently to ring canals in multiple tissues [35]. In both species, SH3 domains of CD2AP/Cindr bind conserved motifs in the N-terminus of Anillin, and Cindr additionally binds the C-terminus of *Drosophila* Anillin (Table 1). Depletion of CD2AP/Cindr leads to binucleation [34], but its role in cytokinesis and whether this role is mediated by an interaction with Anillin are unknown. Since the N-terminal SH3 binding motifs partially overlap with the myosin-binding domain, it is possible that CD2AP competes with myosin for binding and thus affects cytoskeletal organization by Anillin.

2.5 Rho

Human Anillin interacts with RhoA (Table 1) via its AH domain [21]. It is not known if homologues also interact with RhoA, but the AH region throughout metazoa bears some homology with Rhotekin, a well-established RhoA-GTP binding protein. Anillin may regulate RhoA's discrete equatorial cortical localization during cytokinesis, since Anillin also closely associates with the plasma membrane, either directly or via the septins (see 2.3). Anillin and Rho closely colocalize in the contractile ring and the ingressing furrow (Fig. 2). While depletion or inhibition of RhoA blocks contractile ring assembly (and ingression) [36-39], Anillin depletion from most cell types results in much less severe phenotypes (contractile rings form and partially ingress). Depletion of Anillin from *Drosophila* spermatocytes dramatically reduces

equatorial Rho and F-actin [12] and human Anillin is required for a population of endogenous RhoA that persists following TCA fixation, [21, 22]. The highly conserved AH domain in the C-terminus of Anillin is required for stabilizing RhoA localization *in vivo*, and can bind directly to RhoA *in vitro* [21]. Furthermore, Anillin and RhoA can be co-immunoprecipitated, and over-expression of Anillin causes an increase in active RhoA [40]. The dramatic effect of Anillin depletion on Rho localization implies that TCA-fixable Rho does not reflect the entire population of active Rho that promotes contractile ring assembly. Nevertheless, these results suggest that Anillin contributes to the generation of active RhoA and stabilizes it in the cleavage plane.

2.6 Ect2

In further support for the idea that Anillin regulates or stabilizes RhoA localization, Anillin also interacts with Ect2 (Table 1), an activator of RhoA (A. J. Piekny, unpublished observations). This interaction occurs independently of RhoA and requires the AH domain of Anillin and the PH domain of Ect2, which is essential for Ect2's GEF activity [41]. Interestingly, a mutation in the PH region of Ect2 that decreases association with Anillin also decreases Ect2's association with the plasma membrane and the production of Rho-GTP. This suggests that by binding both RhoA and its upstream regulator Ect2, Anillin ensures the efficient production or stable localization of active RhoA in the division plane. It is not known whether these proteins interact simultaneously, sequentially or in a competitive manner. Also, since the Ect2-Anillin interaction appears to be enhanced by Ect2's lipid binding, it is not clear how membrane association contributes to this interaction and to RhoA-GTP production or localization. However, this evidence supports the idea that Rho activation mainly occurs at the membrane. Since Ect2 also binds the Rho GAP Cyk-4 in the central spindle, perhaps the population of Ect2 close to the membrane, and not that in association with the central spindle, generates active RhoA to form the contractile ring. In the absence of Anillin, there may be sufficient Ect2 close to the membrane to generate enough active RhoA to initiate contractile ring formation, however, the ring may become unstable due to insufficient levels (and perhaps uneven distribution) of active RhoA (A. J. Piekny, unpublished observations).

2.7 Cyk-4

Anillin may also determine the division plane through interactions with the central spindle, antiparallel bundled microtubules that form between segregating chromosomes in anaphase (reviewed in [42]). Initially the central spindle is formed deep in the centre of the cell, but it quickly expands to reach the overlying cortex. *Drosophila* Anillin can interact with the central spindle protein Cyk-4 (also known as RacGAP50C (*Drosophila*) and MgcRacGAP (mammalian); Table 1) [20, 43]. The 310 C-terminal amino acids of Anillin directly interact with the N-terminus of RacGAP50C *in vitro* [20]. RacGAP50C also interacts with Ect2 via a domain distinct from its Anillin binding region [20]. In addition, fixed FRET studies demonstrate that Anillin and RacGAP50C directly interact *in vivo* [43]. The central spindle no longer extends to the cortex in Anillin-depleted cells in larval brains [43], and often looks distorted and improperly positioned in human cells [22]. Although it is not clear whether Anillin could bind Cyk-4 that is simultaneously bound to Ect2 and to Pavarotti/MKLP1/Zen-4

(complexed with Cyk-4 as centralspindlin), an exciting possibility is that Anillin helps position the division plane by anchoring the middle of the anaphase spindle to the overlying cortex (Fig. 2; also see review [4]). To date there is no published evidence that Anillin directly interacts with Cyk-4 in other organisms. However, considering the observed pair-wise interactions: Cyk-4-Ect2, Cyk-4-MKLP1/Zen-4, Anillin-Cyk4, Anillin-Ect2, Cyk-4-Rho, Ect2-Rho and Anillin-Rho, it is likely that this signaling module exists in one form or another throughout phylogeny.

2.8 Microtubules

In addition to interactions between Anillin and proteins on the central spindle, there is also evidence that Anillin binds microtubules (Table 1). Anillin was isolated from *Drosophila* extracts on the basis of its affinity for both F-actin and microtubules [44]. Furthermore, the Anillin-rich structures that form following Latrunculin A treatment of *Drosophila* S2 cells often colocalize with the plus ends of microtubules. These peripheral microtubules are required to limit localization of the ectopic structures to the equatorial plane [13]. Regardless of whether the interaction is direct, Anillin–microtubule association may reflect a role for Anillin in communicating the position of the mitotic spindle to the overlying cortex thus ensuring robust contractile ring formation.

2.9 Plasma membrane/lipids

Early in Anillins' history, it was proposed that they could bind lipids of the plasma membrane by virtue of their PH domain, a structurally conserved protein- and lipid-binding motif (Fig. 5) [45]. In support of this function, deletion of the PH region from over-expressed human Anillin constructs prevented Anillin from localizing to the cortex [15, 21]. The high degree of conservation within the PH domain among Anillins also supports the idea that this region has an important, conserved function. However, this region is implicated in binding septins (see 2.3), which themselves can bind lipids; thus, deleting the PH region could affect Anillin's cortical localization by perturbing septin interaction. Currently, it is unknown whether the PH domain of any Anillin can bind directly to lipids.

An amphipathic helix located adjacent to the NLS in the C-terminus (but far from the PH domain) of the *S. pombe* Anillin-like protein Mid1 is necessary and sufficient to direct plasma membrane localization and equatorial enrichment [46]. The membrane targeting activity of the amphipathic helix is increased by Mid1 dimerization. Although it is not known if metazoan Anillins can dimerize (see 2.12), it is possible that Anillins use more than one relatively weak lipid-binding motif (*in cis* or *in trans*) to accomplish high affinity membrane association.

2.10 Clp1/Cdc14

In *S. pombe*, the Anillin-like protein Mid1 interacts with the Clp1/Flp1 phosphatase (related to the budding yeast Cdc14), which counteracts the activities of the mitotic kinase Cdc2, and is crucial for mitotic exit and cytokinesis (see references within [47]). Mid1 was detected by mass spectrometry in a tandem affinity purification of Clp1 and its associated proteins, and this

interaction was confirmed by co-immunoprecipitation, yeast two-hybrid and *in vitro* binding assays [47]. Clp1's recruitment to the contractile ring is dependent on binding to an internal region (amino acids 331-534) of Mid1. Absence of Clp1 in the contractile ring perturbs the stable cortical localization of myosin and Cdc15 (a cytoskeleton-membrane scaffold protein; reviewed in [48]). A low incidence of cytokinesis failure occurs in *clp1* null cells, possibly because the dephosphorylation it catalyses can still occur via another phosphatase. However, Clp1 becomes necessary for the fidelity of cytokinesis when combined with other perturbations, illustrating the requirement for proper kinetics of myosin and other contractile ring structural proteins. Metazoan Cdc14 homologues have been implicated in cytokinesis (reviewed in [49]) but it is not known whether these roles are mediated by an interaction between the phosphatase and Anillin.

2.11 Importins

Drosophila and human Anillins have nuclear localization sequences (NLSs) [9, 15]. One way that an NLS leads to nuclear localization is by binding to nuclear import proteins such as the importins that guide target proteins through nuclear pores (reviewed in [50]). The 400 C-terminal amino acids of *Drosophila* Anillin can directly bind to importins alpha and beta *in vitro* (Table 1) [51]. As is the case for established NLS-importin interactions, RanGTP can disrupt this interaction by competing for importin binding. Mutagenic studies demonstrated that the more archetypal NLS (amino acids 989-999 in *Drosophila* Anillin) was necessary for importin-Anillin binding. These findings may reflect the interphase nuclear localization of *Drosophila* and human Anillins (see section 3; Fig. 2) [9, 15, 22, 40, 52]. However, a second NLS in the N-terminus of human Anillin is the predominant site for nuclear localization [15] and this site is not essential for Anillin's role in cytokinesis [21]. Considering that the *C. elegans* homologues (ANI-1 and ANI-2) lack NLS sequences [19], it is not clear how Anillin's nuclear localization contributes to its function. Anillin's interaction with importins may primarily regulate septin binding rather than nuclear localization.

2.12 Intramolecular interactions

As a cytoskeletal crosslinker and a scaffold of regulatory machinery in the contractile ring, multimerization of Anillin could bring its partners together with higher affinity and/or diversity. Support for the idea Anillin self-associates comes from observations that Anillin truncations (*eg.* lacking the PH domain, or unable to bind Rho) can localize to the cell equator more robustly in the presence of endogenous Anillin than following its depletion ([20]; A. J. Piekny unpublished observations).

If Anillin could dimerize, what functions would this affect? If localization depends on a low-affinity interaction, then dimerization would place two such interaction domains in proximity and thus increase binding affinity. If Anillin binds to Rho, Cyk-4 and Ect2 individually or simultaneously, its dimerization could promote efficient interaction between these partners, and thus Rho GTPase cycling. While F-actin bundling activity resides in a small region of Anillin that is monomeric [9], full-length Anillin bridges F-actin bundles, myosin and septins, and dimerization of Anillin could promote higher orders of cytoskeletal organization.

How could Anillin dimerize? Dimerization is commonly conferred by an extended coiled-coil region that interacts with the sister polypeptide. A small fragment in the middle of *Drosophila* Anillin (amino acids 354-693) is predicted to be 38 kilodaltons (kDa) but has the electrophoretic mobility of a 55 kDa protein, supporting the idea that it has an elongated coiled coil structure [9]. Furthermore, full-length Anillins across phylogeny have a predicted molecular weight close to 130 kDa, but the proteins migrate as multiple bands near 190 kDa by SDS-PAGE, possibly due to adopting an elongated conformation. The COILS algorithm strongly predicts coiled-coil at the beginning of the Anillin Homology domain in *Drosophila*, *C. elegans* (ANI-1), *Xenopus*, mouse and human Anillins, and the mammalian Anillins have additional predicted coils throughout the “middle” of the protein. It would be interesting to test whether deletion of these domains affects Anillin function. A truncation of human Anillin lacking amino acids 460-608, but retaining the coiled coil between residues 700-800, can rescue depletion of endogenous Anillin during cytokinesis [21]. This supports the idea that the coiled coil contributes to Anillin’s function, and suggests that much of the middle of the protein, which has no assigned function, is not essential.

It is also possible that Anillin undergoes intramolecular interactions *in cis*. Perhaps cell cycle phase-dependent modifications (such as phosphorylation; see below) cause Anillin to fold upon itself, inhibiting its anaphase-specific scaffolding activities. Upon entry into anaphase, phosphorylation, dephosphorylation, or competitive interaction with a contractile ring component could alter Anillin’s crosslinking behavior.

S. pombe Mid1 sediments through a sucrose gradient as large oligomers [46], but no other Anillins have been directly demonstrated to self-associate. As researchers continue to study Anillin in cells using functional and dysfunctional transgenes, it is important to know whether exogenous and endogenous Anillin interact. The knowledge of whether Anillin self-associates will also be valuable for computational modeling of the contractile processes that Anillin seems to coordinate.

2.13 Anillin as a scaffold

From the interaction studies enumerated above, Anillin appears to be a very busy and very crowded protein (Table 1). The evidence that it associates with so many partners fosters hypotheses that Anillin bridges these partners to each other, coordinating them in time and space (Fig. 2). Anillin itself is not known to form filaments *per se*. So does, or could, single Anillin polypeptides bind all of these partners simultaneously?

Anillin can indeed function as a cytoskeletal crosslinker, coupling septin filaments to F-actin *in vitro* [10]. Importantly, Anillin, and not other F-actin bundling proteins, promoted the association of septins with F-actin. In mammalian cultured cells, over-expression of an Anillin fragment displaced septins from actomyosin stress fibers and disrupted their organization [10]. These results suggest that septins contribute to F-actin organization, and this function requires a linker protein such as Anillin.

Fluorescence/Förster resonance energy transfer (FRET) studies with immunolabeled Anillin and Phalloidin-labeled F-actin demonstrated a close physical interaction *in vivo* [43]. The same method revealed that Anillin also contacts GAP50C (MgcRacGAP/Cyk-4; see 2.7). A lack of FRET between GAP50C and F-actin suggests that their interactions with Anillin are

mutually exclusive. Alternatively, they may interact with Anillin simultaneously, but due to their orientation, be outside the reach of FRET.

In addition to facilitating nuclear localization of Anillin (see 2.11), importins also regulate Anillin's capacity to crosslink and coordinate cytoskeletons. Binding of *Drosophila* Anillin to the septin Peanut (but not Sep2) *in vitro* was inhibited by the inclusion of excess importins [51]. In embryos, this competition does not displace Anillin or Sep2 from metaphase furrows, but disrupts their organization there and blocks the recruitment of Peanut. This importin-septin competition suggests that in regions of high concentrations of RanGTP, such as in the nucleus or in the vicinity of chromatin during mitosis, Ran binds importins, freeing Anillin to bind septins and organize cortical structures. Conversely, high levels of septins, which occurs in some cancers [53] could out-compete importin binding and promote retention of Anillin in the cytoplasm.

3. Cell cycle regulation

The localization of Anillin changes dramatically throughout the cell cycle, reflecting either multiple functions for Anillin, or a necessity to sequester it during some stages of the cell cycle (Fig. 2). In interphase *Drosophila* and human cultured cells and in *S. pombe*, Anillin localizes primarily to the nucleus (Fig. 2) [9, 15, 22, 54]. In early embryos of *Drosophila* and *C. elegans*, Anillin does not enter the nucleus during interphase. Instead, it participates in specialized contractile events: metaphase furrows during syncytial divisions in *Drosophila* (Fig. 1; [7, 9]) and ruffles and the pseudocleavage furrow during polarity establishment of the *C. elegans* zygote [19].

During mitosis, concurrent with nuclear envelope breakdown, Anillin localizes to peripheral stress fibers in mammalian cells [10], where it may contribute to their disassembly, and to the increasingly round cortex of *Drosophila* cells [9, 13, 43]. After anaphase onset, Anillin rapidly accumulates at the equatorial cortex where it colocalizes with contractile ring components including RhoA, actin, myosin and the septins (Fig. 2). Anillin remains enriched in the furrow throughout ingression and accumulates in the midbody (Fig. 2). During cellularization (Fig. 1) of the *Drosophila* syncytial blastoderm, nuclei are compartmentalized by furrows that descend into the interior of the embryo. Anillin localizes to the leading edges of this cytokinetic furrow-related structure and is required for the proper rate and extent of its ingression [8, 9, 51].

In G1 phase, human and *Drosophila* Anillin relocates to the nucleus and is targeted by the APC^{Cdh1} for degradation by the proteasome (Fig. 2) [9, 22, 34]. Ubiquitination occurs at a D-box at the very N-terminus of human Anillin [22]. Similarly, *S. pombe* Mid2 is degraded after mitotic exit [29]. Human Anillin's interaction with CD2AP may play a role in ubiquitination because CD2AP can also bind to a ubiquitin ligase [34]. Throughout the remainder of the cell cycle, levels of nuclear Anillin steadily increase [9, 34]. Anillin is thought to be absent from cells that have exited the cell cycle [9, 55] but has been detected in the nucleus and cytoplasm of many types of terminally differentiated human cells [52].

Anillin's varying localization throughout the cell cycle suggests that it is regulated by phosphorylation in a cell cycle phase dependent manner. Human Anillin undergoes mitosis-specific phosphorylation that manifests as retarded electrophoretic mobility and can be reversed by treatment with lambda phosphatase [9, 34]. However, it is not known what kinase(s) makes these modifications. *S. pombe* Mid1 is hyperphosphorylated as it exits the nucleus [54]; the Polo

kinase Plo1 is implicated in this event [56]. *S. pombe* Mid2, which acts later in cytokinesis, is also phosphorylated [29]. Human, *Drosophila* and *C. elegans* Anillins all contain putative sites for Cdk1, Plk1 and Aurora B and 16 phosphorylation sites in *C. elegans* ANI-1 were detected by mass spectrometry (A. S. Maddox, unpublished observations). Since phosphorylation can affect protein conformation, binding partners and activity, phosphorylation of Anillin could regulate its interactions with the components of the cytokinetic machinery discussed above.

4. Outstanding questions about Anillin

4.1 How does Anillin get to the equator?

In *S. pombe*, Mid1 translocates from the centered nucleus to the overlying cortex where it marks the cell equator [57]. Since metazoan Anillins also cycle from the nucleus in interphase to the contractile ring in cytokinesis (Fig. 2), it is tempting to suggest that the above concept also applies in other systems. However, in metazoans, the anaphase spindle, and not the premitotic nucleus, dictates the division plane. Furthermore, Anillin can localize to Rappaport furrows in fused mammalian cells, where there has been no chromatin [15]. Anillin also localizes to asymmetrically positioned furrows of mono-astral cells stimulated to enter anaphase, in which it is unlikely that the chromatin positions the division plane [58].

Anillin recruitment to the cell equator depends on Rho and its activator Ect2 in *Drosophila* and human cells [13, 21, 22, 59]. Human Anillin bears homology to Rhotekin [21]; thus, Anillin is likely a contractile ring-specific Rho effector. Treatment with Latrunculin A or depletion of formins demonstrated that F-actin is required for normal Anillin recruitment [13, 14]. However, in *Drosophila* spermatocytes, Anillin localizes to the site of division prior to the detectable enrichment of actin [11]. Myosin is not required for Anillin localization to the contractile ring [13, 14, 18, 19]. Furthermore, deletion of either or both the actin- or myosin-binding domains does not abolish Anillin localization [15, 21]. Therefore, in metazoa, Rho is likely the primary recruiting factor for Anillin.

4.2 What are the roles of Anillin in cytokinesis?

S. pombe Mid1 has the ultimate early role: it localizes to the equatorial cortex before any other contractile ring component and is required for every other component to localize [56]. Mid1 initially forms “nodes,” onto which other contractile ring proteins are added, which coalesce to form the contractile ring [57] and Mid1 is lost from the equatorial cortex before the actomyosin ring constricts [60]. However, in mid1 mutants, aberrant contractile rings can assemble [54, 56, 60]. The finding that contractile ring assembly defects in mid1 mutants can be rescued by signaling from the SIN (septation initiation) pathway [61] underscores that Mid1 acts in temporal coordination of cytokinesis with chromosome segregation. Furthermore, Mid1 is dispensable for ring positioning if cell-wall deposition is blocked [62]. Thus, Mid1 may normally help center the ring during its assembly, optimizing contractility to prevail in a tug of war between the cytoskeleton and membrane anchoring to the septum.

Metazoan Anillins act early in cytokinesis to coordinate contractile ring assembly and organization. As described above, Anillin crosslinks myosin, septins and F-actin in contractile structures (Fig. 1-4; see 2.1-2.3) [8, 12, 13, 19, 20, 63].

Interestingly, some of Anillin's roles in cytokinesis can be compensated for by unrelated mechanisms. Human Anillin becomes crucial for equatorial enrichment of F-actin and myosin (and thus furrowing) when the central spindle is perturbed by depletion of the MKLP1 component of centralspindlin [21]. This result suggests that Anillin and the spindle midzone function redundantly, perhaps in equatorial specification or activation of contractility. *C. elegans* Anillin (ANI-1), normally dispensable for successful cytokinesis, is required for full furrow ingression when the homologous kinesin, ZEN-4, is depleted [23]. Furthermore, *C. elegans* Anillin (ANI-1) is required for asymmetry within the division plane, which makes cytokinesis robust to perturbations of contractility [24]. *Drosophila* spermatocytes suffer less binucleation following Anillin depletion if cadherins are over-expressed [12]. This phenomenon has been attributed to the shared ability of Anillin and adherens junctions to bundle F-actin.

Inhibition of Anillin by injection of an anti-Anillin antibody in mammalian cells slowed the rate of ring constriction throughout the course of ingression [15]. Depletion of Anillin from human or *Drosophila* cultured cells leads to lateral oscillation of the cytokinetic furrow, and eventual failure [12, 13, 18, 20, 22]. Therefore, in these systems actomyosin contractility still occurs, but cannot be accurately maintained at the division plane. While the ingression rate has not been measured following Anillin depletion except in *C. elegans* (where it is normal; [24]), the apparent discrepancy between the antibody injection study and the RNAi experiments could reflect that the antibody crosslinks Anillin rather than completely removes its function. In *Drosophila* embryos, Anillin C-terminal point mutants display abnormal kinetics of ingression of cellularization furrows [8]. Cellularization relies on the persistent addition of membrane surface area (which increases 25 fold during this event). Therefore, Anillin's requirement in this process may reflect a role in organizing membrane dynamics via its interactions with septins, rather than modulating contractility. It is also possible that Anillin specifically coordinates membrane dynamics and contractility, coupling the contractile ring to lipids or curvature specific to the cell equator.

Anillin may also act late in cytokinesis. Constriction of the contractile ring leads to formation of the midbody (also known as the Flemming Body), an electron dense structure containing components of the central spindle, contractile ring, and membrane trafficking machinery [64]. Failure to generate this minute cell-cell bridge allows fusion of the nascent daughter cells and binucleation. Depletion of Anillin from *Drosophila* cultured cells revealed a role in stabilizing the midbody, since depleted cells displayed reduced midzone microtubule integrity and extensive blebbing around the midbody [59, 65]. During the regulated cytokinesis failure of postnatal cardiomyocytes, Anillin is poorly focused at the midbody, further suggesting that its proper localization at this late stage is important for cytokinesis completion [55]. Importantly the RNAi results likely reflect incomplete Anillin depletion, as subsequent RNAi-based studies in *Drosophila* S2 cells revealed defects before the midbody stage [13]. It is possible that Anillin acts both early in organizing the contractile ring, and later to stabilize the midbody and facilitate abscission.

4.3 Does Anillin have roles outside of cytokinesis?

While Anillin is thought to specifically localize to contractile rings [9], there is some evidence that it participates in events other than cytokinesis. Anillin is abundant in the cytoplasm of diverse human tissues, both post-mitotic and self-renewing [52], but its roles there are completely uncharacterized.

Anillin exits the nucleus of mammalian cultured cells at the onset of mitosis and localizes to stress fibers as they disassemble during mitotic cell rounding [10]. Furthermore, Anillin over-expression can either promote the assembly of [40] and to disrupt stress fibers [10]. The increased motility of cells over-expressing Anillin [40] suggests that it can promote turnover of interphase actomyosin structures. By contrast, Anillin is highly enriched in the Z discs of myocardial cells, where myosin and actin are stably anchored [52]. Human Anillin is strongly expressed in neural tissues [52] where its protein levels are high in the nucleus and cytoplasm, but its role in neurons is not understood. As in the contractile ring, in these other contexts Anillin may act 1) by promoting a certain type of cytoskeletal organization or 2) by coordinating the actomyosin cytoskeleton with the Rho activation module.

4.4 In species with more than one Anillin isoform, is there competition or collaboration among them?

In *C. elegans* there are three genes with homology to Anillins, encoding a C-terminal PH domain and a stretch of homology upstream (the Anillin homology domain). All three genes are transcribed, but functions are only known for the two longer isoforms: ANI-1 and ANI-2. ANI-1 is highly expressed in early embryos and important for cytoskeletal organization during polarity establishment and cytokinesis [19, 23]. ANI-2 is present at very low levels in embryos, and is enriched in the oogenic gonad where it is crucial for its syncytial structure and oocyte production [19]. The abundance and function of ANI-1 and -2 have not been explored for any other tissues of the worm, but due to similarities in their C-termini, they may compete for binding partners when both isoforms are present.

Drosophila has only one gene for Anillin (*scraps*), but this gene has at least three different transcripts. A and B isoforms are similar in size and differ via an alternatively spliced exon in their N-terminus (Flybase). Isoform C is identical in its N-terminus to isoform B, but lacks the C-terminal half of the protein. Human Anillin is predicted to produce many alternatively spliced products (8 to 14; Ensembl). Some splice forms are predicted to encode only regions that mediate interaction with RhoA, Cyk-4, Ect2 and the septins. Splice variants lacking NLSs would be constitutively cytoplasmic and could alter RhoA activation cytoskeletal organization in interphase and their expression may compete with full-length Anillin for binding partners. It is currently unknown whether the expression of alternative splice forms correlates with differences in tissue or cell types, with quiescent versus renewing cells, or with the prognostic stage of cancers. The observations that various *Drosophila* Anillin point mutants (Fig. 5) fail in cellularization versus conventional cytokinesis support the idea that certain sequences within Anillin are differentially required among contractile events [8]. Furthermore, a collection of Anillin truncations expressed in human cells can rescue different aspects of the Anillin depletion phenotype [21].

In *S. pombe*, Mid1 and Mid2 localize to the contractile ring during mutually exclusive time intervals, and have completely distinct roles. Mid1 marks the equatorial location of contractile ring assembly and is required for recruitment of all other ring proteins (see 4. 2) [57].

Mid2 appears as Mid1 departs, after ring assembly is complete and before ingression [60]. Therefore, these related Anillin homologues are unlikely to directly interact or compete for interactors.

The identity of Anillin homologues in budding yeast is unresolved. Notably, a role for Anillin in specifying or maintaining identity of the division plane (as is implicated for fission yeast, *Drosophila* and human Anillins) is less relevant because the division plane of *S. cerevisiae* (the bud neck) is established long before cytokinesis. Boi1 and Boi2 are closely related proteins that have C-terminal PH domains and localize to the division plane [66, 67]. These two potential Anillin homologues function redundantly [68]; the presence of two related proteins in this system is likely to simply reflect a recent gene duplication and not a diversification of function.

4.5 Does Anillin act in cancer development or progression?

It is not surprising that as a key regulator of cytokinesis, Anillin has been linked to cancer. Profiling diverse human tumours revealed that with the exception of brain tumours, Anillin expression was upregulated from ~2 – 6 fold. Furthermore, higher Anillin expression levels correlated with the metastatic potential of tumours [52]. A separate study showed that Anillin was expressed at 20-fold higher levels in pancreatic cancers than in normal pancreatic tissue [69]. While these studies are correlative, they suggest that Anillin over-expression leads to increased cytosolic levels in interphase cells, where it could cause cell shape changes and cell motility that contribute to metastasis. Consistent with this hypothesis, over-expressed Anillin fragments localize to ectopic foci that contain RhoA and septins [15, 21]. Anillin over-expression in lung cancer cell lines leads to increased levels of active RhoA and cell motility [40]. Anillin may also have functions in the nucleus that have not been identified, and over-expression of Anillin could also affect this function. In support of this idea, increased nuclear levels of Anillin have been correlated with poor tumour prognosis [40]. Importantly, the contractile ring in which Anillin is known to act is an exquisitely regulated organelle that must be incredibly dynamic in order to function properly. It is possible that abnormal Anillin expression is pathogenic simply by affecting the fidelity of cytokinesis.

Figure legends

Table 1. The structure of human Anillin is shown, illustrating its protein-protein interaction domains (NLS sequences are shown as black bars in the N-terminus). The amino acids are numbered according to the species used. The interacting proteins are indicated along with the system, the method used and the region of Anillin they interact with. The hypothesized roles of these interactions are indicated along with the original references.

Fig. 1. A schematic of cellularization of the *Drosophila* embryo syncytial blastoderm. The concerted action of a network of rings and of membrane addition establish plasma membrane (green) partitions among all the nuclei (blue). Contractile ring proteins (red) localize to the cellularization “front,” and eventually to discrete ring canals connecting each nascent cell to the yolk in the center of the embryo. A *Drosophila* embryo is shown that has almost completed cellularization, bearing fluorescently-tagged Anillin on the cellularization front. A maximum

intensity projection of several microns of thickness is shown. The red box represents the region depicted above. Image courtesy of Gilles Hickson.

Fig. 2. A schematic of mammalian cells depicting the dynamic changes in Anillin localization and its hypothesized interactions through the cell cycle stages. In S/G2, Anillin accumulates in the nucleus, likely through interactions with importins. During anaphase, Anillin accumulates in the contractile ring (shown in transparent red). A high-resolution view of Anillin's interactions with other cytokinesis proteins are shown in the hatched box. At the end of mitosis Anillin re-enters the nucleus, and is degraded in G1. All proteins depicted are annotated in the figure key.

Fig. 3. Depletion of Anillin (ANI-1) from *C. elegans* zygotes results in abnormally symmetric contractile ring closure. Left: The contractile ring, labeled with GFP-tagged myosin, is viewed as if from one pole of the anaphase spindle. Right: The position of the contractile ring is plotted for each point in a time course (elapsed time ~5 minutes; green = early; red = late). Images courtesy of Li Zhang and Jonas Dorn.

Fig. 4. Kymographs show the changes in localization of GFP tagged myosin over time, as cleavage furrows form and ingress in control and Anillin depleted HeLa cells. In control cells, myosin flows into and enriches in the equatorial plane and later moves into the polar regions of the cell. In Anillin-depleted cells myosin initially accumulates in the equatorial plane, but then relocalizes outside the equator, oscillating dramatically from pole to pole. Kymographs are maximum intensity projections along the axis of the pink lines. The scale bar is 5 μm . Time elapsed: 16 minutes.

Fig. 5. A ribbon diagram of the predicted structure of the PH domain from human Anillin is shown. Also shown is an alignment of the amino acid sequence of the PH domain between human and *Drosophila* Anillin. Mutations characterized in *Drosophila* are indicated [8]. Image courtesy of Lauren Narcross.

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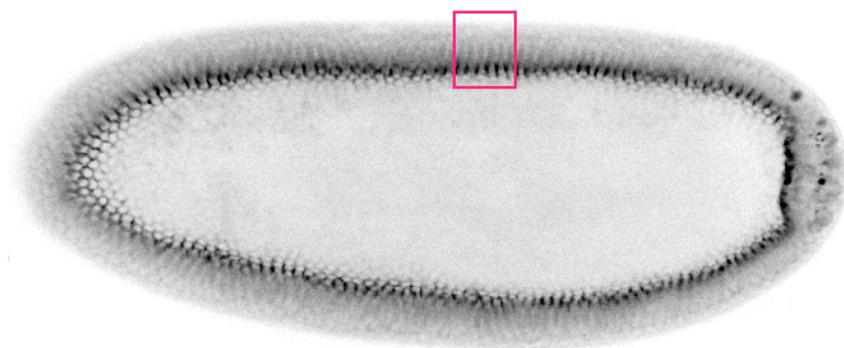
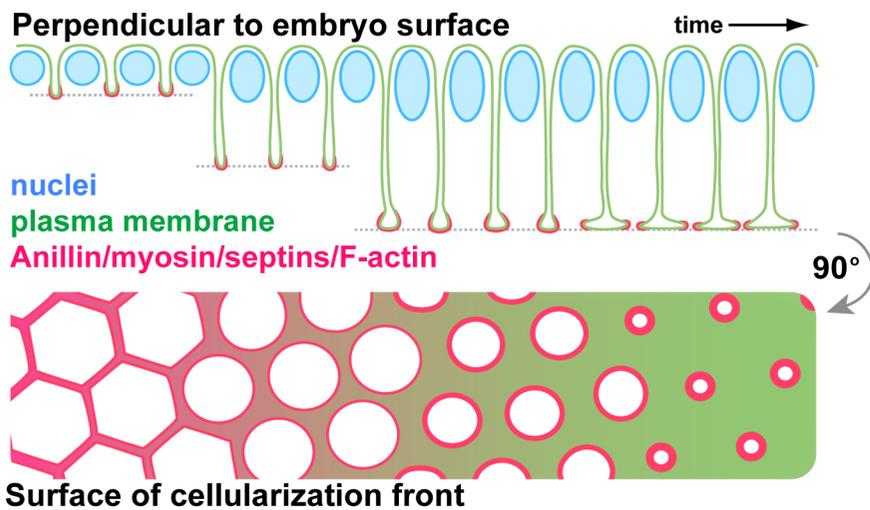
Hs Anillin



980 1124*

*numbering corresponds to the longer alternatively spliced human isoform. All other numbering matches the species-specific isoform.

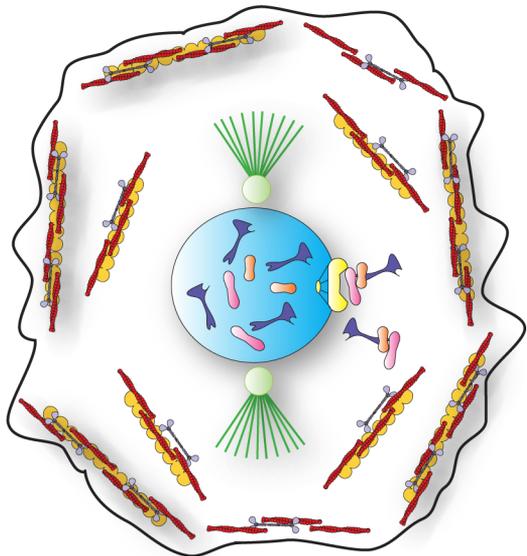
Protein	System	Method	Region	Role	References
APC/Cyclosome	Human (HeLa S3 cells)	-35S-labeled anillin was ubiquitinated with immunopurified APC/C Cdh1, co-IP Cdh1 with Anillin in early G1	 1 45 D Boxes (16-19; 41-44)	cell cycle dependent degradation of Anillin	Zhao & Fang, 2005
mDia2	Human (HeLa/HEK-293 cells)	-pull-down of Anillin from lysates using bacterially-expressed mDia2, co-IP using over-expressed mDia2 fragments, <i>in vitro</i> binding	 1 91	crosslink Actin regulator with contractile ring	Watanabe et al., 2010
CD2AP	Human	-yeast-two-hybrid, <i>in vitro</i> binding, immunoprecipitation	 1 155	intercellular bridge stability	Monzo et al., 2005
	<i>Drosophila</i>	-pull down Cindr from lysates using bacterially-expressed Anillin	 1 328 930 1239		
Nonmuscle myosin II	<i>X. laevis</i>	-myosin pulled down from <i>Xenopus</i> extract (cytochalasin D-treated) with immobilized Sf9-purified Anillin, pull down of purified myosin (phosphorylated via MLCK) via bacterially purified Anillin	 142 254	stabilize myosin localization at equatorial plane	Straight et al., 2005
F-actin	<i>Drosophila</i>	-co-sedimentation, binding to phalloidin-stabilized actin filaments, actin filament bundling	 127 258 340 371 binding bundling	furrow ingression crosslinker for F-actin in furrows	Field et al., 1995
	Human	-co-sedimentation with bacterially-expressed proteins	 231 454 binding		
	<i>X. laevis</i>	-F-actin stabilized with phalloidin was bundled by Anillin purified from Sf9 cells, cosedimentation assay	 255 418 bundling binding		
	<i>Drosophila</i>	-fixed FRET endogenous Anillin and F-actin	Full-length		
RacGAP50C	<i>Drosophila</i>	-Fixed FRET between Anillin and GAP50C, yeast two hybrid	 517 1212	crosslink the central spindle to the contractile ring	Gregory et al., 2008
	<i>Drosophila</i>	-affinity purification with immobilized Anillin and mass spec, <i>in vitro</i> binding	 930 1239		
RhoA	Human (HeLa/HEK-293 cells)	-pull down RhoA from lysates using bacterially-expressed Anillin, <i>in vitro</i> binding	 645* 980* 874-880 contribute to binding	stabilize RhoA & crosslink RhoA to the actin/myosin contractile ring	Piekny & Glotzer, 2008
Ect2	Human (HeLa/HEK-293 cells)	-pull down Ect2 from lysates using bacterially-expressed Anillin	 926* 980*	stabilize RhoA activation and localization	A.J. Piekny (unpublished observations)
Importin $\alpha\beta$	<i>Drosophila</i>	- <i>in vitro</i> binding, competes with peanut binding to Anillin (not Septin 2)	 815 989 999 1201 NLS	regulate nuclear localization, modulate septin binding	Silverman-Gavrila et al., 2008
Septin (Hcdc10/Sept7)	Human (BHK cells)	-ectopic recruitment into foci via over-expression Anillin fragments (localization)	 929 1124	furrow ingression, septin recruitment and/or stabilization	Oegema et al., 2000
Septins (Peanut, Sep 2 & 5)	<i>Drosophila</i>	-affinity purification with immobilized Anillin and mass spec	Full-length		
Septins (Peanut & Sep 2)	<i>Drosophila</i>	-pull down from embryo lysate using bacterially-expressed Anillin	 815 1201		
Septins (Sept 2, 6 & 7)	Human	-Anillin-bundled F-actin recruited septin complex	 748 1116		
Microtubules	<i>Drosophila</i>	-cosedimentation with taxol-stabilized microtubules, after Anillin was affinity purified with an F-actin column	Full-length	crosslink actin, septin and microtubule cytoskeletons?	Sisson et al., 2000



YFP-tagged Anillin during cellularization

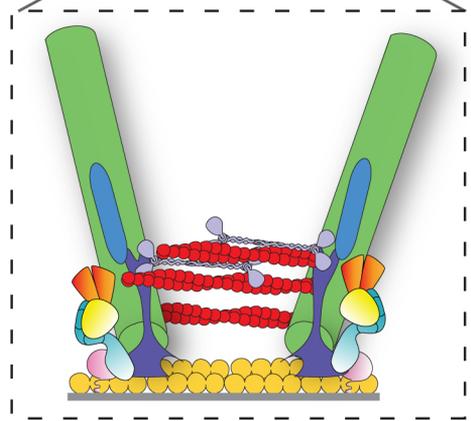
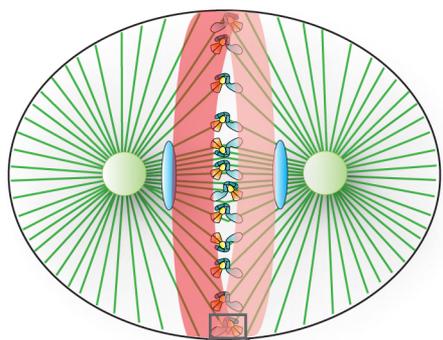
S - G2

Anillin accumulates in the nucleus



Mitosis - Anaphase

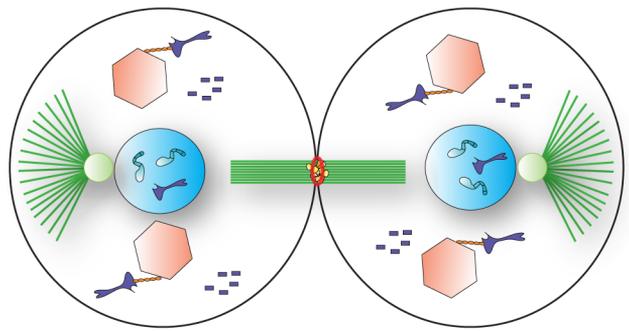
Anillin accumulates in contractile ring

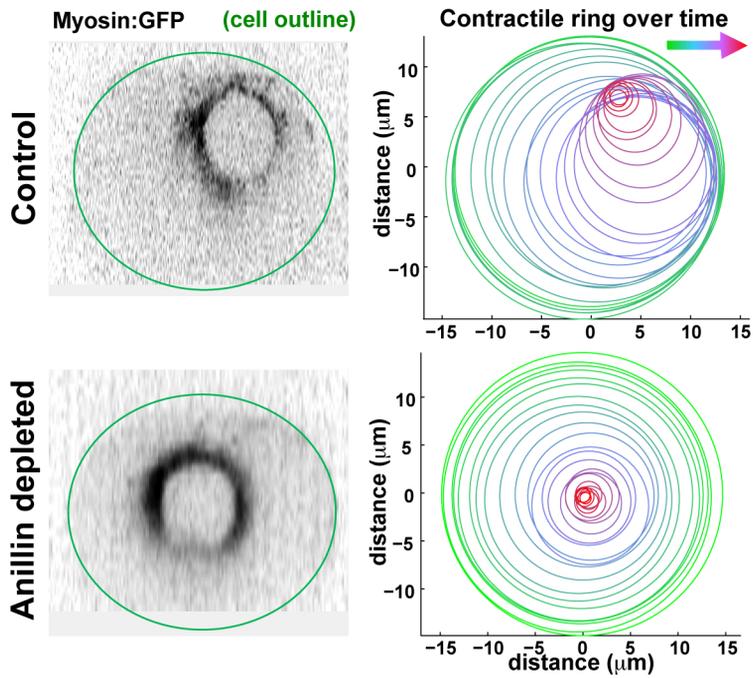


- | | | | |
|--|-------------------|--|-------------|
| | Active RhoA | | Cyk-4 |
| | Ect2 | | Tubulin |
| | MKLP1 | | Nucleus/DNA |
| | Actin | | Myosin |
| | Septin | | NPC |
| | Importin α | | CD2AP |
| | Importin β | | APC Cdh1 |
| | Anillin | | Ubiquitin |

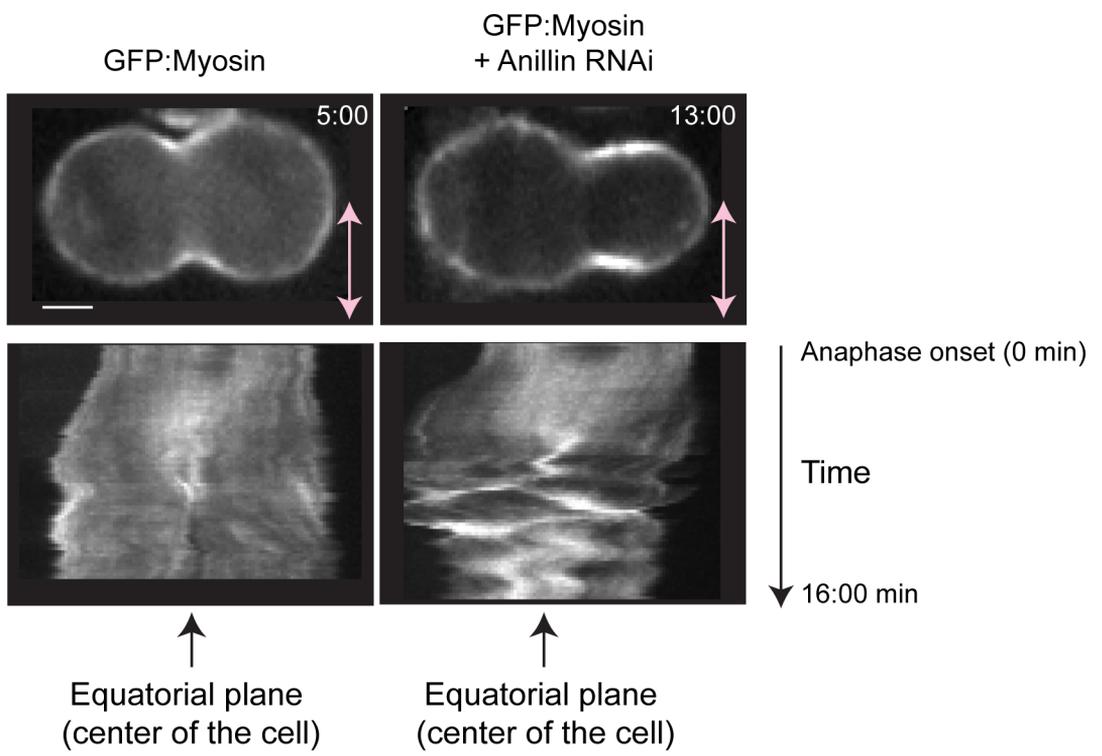
End of mitosis - G1

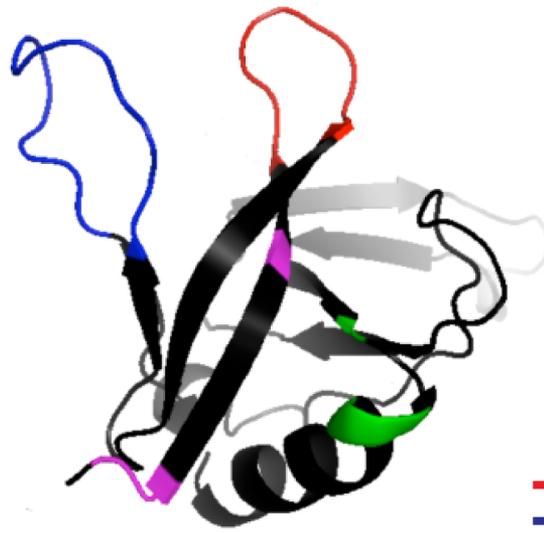
Anillin re-enters the nucleus and is targeted for degradation





Piekny & Maddox Figure 3





- phospholipid loop β 1/2
- phospholipid loop β 3/4
- part of α -helix

