How to scaffold the contractile ring for a safe cytokinesis – lessons from Anillin-related proteins

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Summary

The ingression of a cleavage furrow separates the two daughter cells at the end of cell division. In many organisms this furrow ingression is driven by the assembly and contraction of actomyosin filaments, forming a contractile ring. To achieve a successful cytokinesis, these actomyosin filaments need to be assembled in an organized manner. For this purpose, a network of cytoskeletal proteins is built at the cleavage site to act as a scaffold for actomyosin filaments and to connect them to the plasma membrane. The *Drosophila melanogaster* protein Anillin, and its related proteins in other organisms, has a pivotal role in the organization of this scaffold in many species, ranging from yeast to humans. Recent studies indicate that Anillin- **related proteins interact not only with the structural components of the contractile ring, but also with the signalling factors that control their dynamics. In addition, *Drosophila* Anillin connects the actomyosin ring to the spindle microtubules through its interaction with the RacGAP component of the centralspindlin complex. Here I review the structures and functions of Anillin and Anillin-related proteins in various model systems, and aim to highlight both the common and distinctive features of these essential organizers of the molecular machinery that drives furrow ingression.**

Key words: Actin, Cleavage furrow, Cytoskeleton, Myosin, RhoA

Introduction

During cytokinesis, which occurs at the end of cell division, a cleavage furrow forms between the segregating chromosomes and ingresses to bisect the dividing cell (D'Avino et al., 2005; Eggert et al., 2006). Although the mechanisms that are responsible for furrow positioning vary considerably from yeast to metazoans (Balasubramanian et al., 2004; Barr and Gruneberg, 2007; Guertin et al., 2002), current models propose that furrow ingression is always driven by the assembly and contraction of actomyosin filaments that organize into a contractile ring (Fig. 1A), a very dynamic structure in which filaments are continuously assembled and disassembled. In many organisms, the basic machinery for assembling the contractile ring is similar in that it includes actin, myosin and their regulatory factors. By contrast, the signalling pathways that control assembly and contraction of the ring differ considerably among organisms. These mechanisms and pathways have been recently reviewed in detail (Balasubramanian et al., 2004; Barr and Gruneberg, 2007; D'Avino et al., 2005; Eggert et al., 2006) and are beyond the scope of this article. In this Commentary, I focus on the scaffolding process that facilitates the construction of the actomyosin ring in metazoans and fission yeast.

To achieve successful cytokinesis, actomyosin filaments are assembled upon a network of cytoskeletal proteins (including septins) at the cleavage site that acts as a scaffold and might connect the filaments to the plasma membrane. One of the proteins that has a pivotal role in the organization of this scaffold is Anillin, which was first identified 20 years ago through F-actin affinity chromatography of *Drosophila melanogaster* embryonic extracts (Miller et al., 1989). Since then, Anillin-related proteins have been identified in many organisms, from yeasts to humans, and it has been shown that they are essential for proper assembly of the contractile ring and successful cytokinesis. More recent studies have also indicated that Anillin is not only necessary for the organization

and/or recruitment of structural components of the ring, but also for linking these components to signalling proteins that control cytokinesis and mitotic exit (Clifford et al., 2008; D'Avino et al., 2008; Gregory et al., 2008; Hickson and O'Farrell, 2008; Piekny and Glotzer, 2008). Moreover, Anillin establishes a direct connection between the contractile ring and spindle microtubules at the cell division site in *Drosophila* (D'Avino et al., 2008; Gregory et al., 2008). Finally, the human homologue of Anillin is overexpressed in diverse human tumours and has a key role in lung carcinogenesis (Hall et al., 2005; Suzuki et al., 2005).

Despite its widespread role in the organization of the contractile ring, there are several interspecies differences in the structure and function of Anillin-related proteins. In this article, I review the structure and function of Anillin-related proteins in various model systems, and aim to highlight both their common and distinctive features. I also describe the different mechanisms that control localization of Anillin-related proteins and analyze the latest findings regarding the role of these proteins in the assembly of the contractile ring. Finally, I discuss the possible evolutionary pathways of Anillin-related proteins, and potential future research directions for these proteins.

Mechanisms of contractile-ring formation in yeast and metazoans

In animal cells, the small GTPase RhoA is a key regulator of both the assembly and contraction of actomyosin filaments (Piekny et al., 2005). RhoA promotes F-actin polymerization and the activity of the non-muscle myosin II (Fig. 1B), and its activation represents a key step in furrow formation and ingression. A Rho guanine-nucleotide-exchange factor (RhoGEF), known as Pebble (Pbl) in *Drosophila* and ECT2 in mammals, activates RhoA at the cleavage site (Kimura et al., 2000; Prokopenko et al., 1999; Tatsumoto et al., 1999). Evidence in both flies and mammals indicates that activity

of Pbl/ECT2 requires it to interact with the Rho-family GTPase-activating protein (GAP) known as RacGAP50C in *Drosophila* and MgcRacGAP in mammals (Fig. 1B) (Kamijo et al., 2006; Nishimura and Yonemura, 2006; Somers and Saint, 2003; Yuce et al., 2005; Zhao and Fang, 2005b). This RacGAP molecule is one of the two members of an evolutionarily conserved complex, dubbed centralspindlin (Mishima et al., 2002), the other component being a plus-end-directed kinesin-like motor protein that is termed ZEN-4 in *C. elegans*, Pavarotti (Pav-KLP) in *Drosophila* and MKLP1 in mammals (Adams et al., 1998; Nislow et al., 1992; Powers et al., 1998; Raich et al., 1998). Because of the activity of its motor component, centralspindlin rapidly accumulates at the plus-ends of two populations of microtubules in anaphase – the central spindle, which consists of an array of interdigitating and antiparallel filaments, and the peripheral or equatorial microtubules, which are a particularly stable subset of astral microtubules that contact the cortex at the future cleavage site (Fig. 1A). Thus, cleavage-furrow ingression appears to require a signal from the spindle microtubules in the form of a RacGAP signalling molecule. Consistent with this, RacGAP50C and MgcRacGAP are necessary for furrowing in flies and mammals, respectively (D'Avino et al., 2006; Kamijo et al., 2006; Nishimura and Yonemura, 2006; Somers and Saint, 2003; Yuce et al., 2005; Zhao and Fang, 2005b) and mislocalization of RacGAP50C along the plasma membrane can provoke ectopic furrowing in *Drosophila* cells (D'Avino et al., 2006).

The fission yeast *Schizosaccharomyces pombe* divides by medial cleavage after forming a centrally placed actomyosin-based contractile ring (Balasubramanian et al., 2004; Guertin et al., 2002). F-actin, which concentrates in patches at the growing ends of the cell in interphase, relocates to an equatorial cortical ring in metaphase. This ring thickens during anaphase and contracts concomitantly with septum formation. At the end of furrow ingression, digestion of the septum separates the two daughter cells. In contrast to microtubules in metazoans, microtubules in *S. pombe* do not have a direct role in the placement of the actomyosin ring; instead, the position of the nucleus determines the cleavage site (Chang and Nurse, 1996). However, nuclear positioning in the cell centre does depend on microtubules and on their interaction with both the spindle-pole bodies (SPBs) and cytoplasmic interphase microtubule-organizing centres (iMTOCs) (Hagan and Yanagida, 1997; Tran et al., 2001).

Domain structure and interactions of Anillin-related proteins

Anillin was first identified in *Drosophila* on the basis of its affinity for F-actin, but related proteins have been discovered in all eukaryotes. *Drosophila* Anillin contains three potential nuclear localization signals (NLSs), two Src-homology 3 (SH3)-binding consensus sequences and a pleckstrin-homology (PH) domain (Rebecchi and Scarlata, 1998) at its C-terminus (Fig. 2) (Field and Alberts, 1995). The actin-binding and actin-bundling domain of Anillin is located in the N-terminus (Fig. 2; Table 1) (Field and Alberts, 1995). A region in the C-terminus that includes the PH domain shows high similarity to related proteins in other organisms, and is known as the Anillin homology region (AHR) (Fig. 2) (Oegema et al., 2000). Studies in vertebrates have identified a conserved region that can interact with non-muscle myosin II and is located upstream of the actin binding and bundling domain (Fig. 2) (Straight et al., 2005).

Homology searches identified three Anillin-related proteins in *C. elegans*, ANI-1, ANI-2 and ANI-3. All three proteins have a
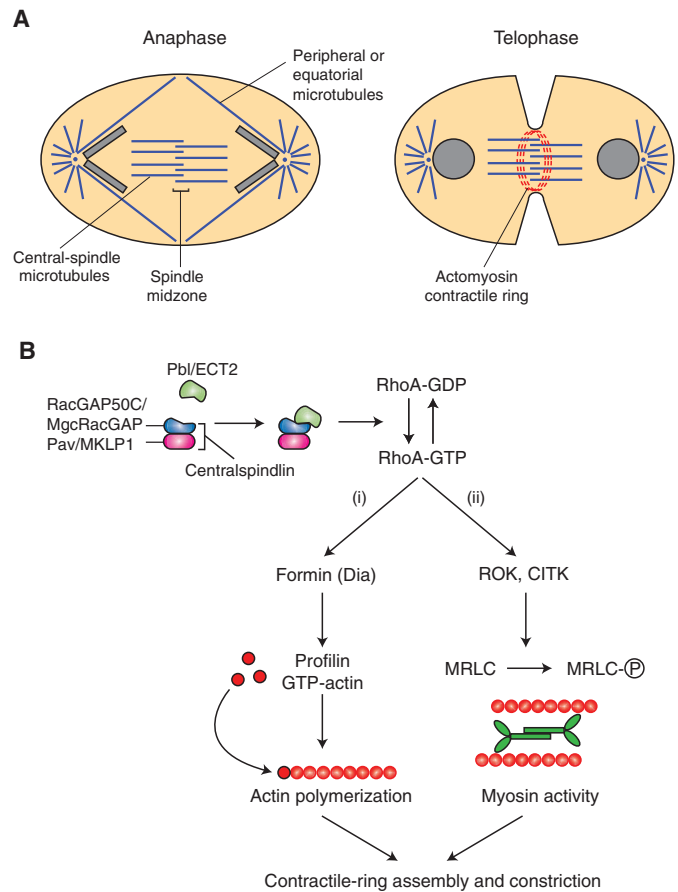

Fig. 1. Mechanisms and signalling pathways that govern furrow formation and ingression in animal cells. (A) Schematic representation of an animal cell in anaphase and telophase. Chromosomes and nuclei are in grey, microtubules in blue and actomyosin filaments in red. The central spindle, spindle midzone and contractile ring are indicated. (B) RhoA activation at the cleavage site during cytokinesis in *Drosophila* and vertebrates. Interaction between the RacGAP component of the centralspindlin complex and the RhoGEF Pbl/ECT2 induces the formation of active, GTP-bound RhoA, which in turn promotes (i) profilin-mediated actin polymerization through the family of formin-homologous proteins such as Diaphanous (Dia); and (ii) phosphorylation of the myosin regulatory light chain (MRLC) by two kinases, Rho kinase (ROK) and Citron kinase (CITK). Together, these events promote the sliding of myosin heads along actin filaments, and therefore the formation and ingression of the cleavage furrow.

C-terminal AHR with a PH domain, but only ANI-1 contains sequences towards the N-terminus that are similar to the myosin- and actin-binding domains that are observed in humans and vertebrates (Fig. 2) (Maddox et al., 2005).

The human genome encodes a single Anillin homologue (anillin), which contains a conserved AHR with a PH domain, a consensus SH3 binding site and several potential NLSs (Fig. 2). However, only the three potential N-terminal NLSs are necessary for nuclear localization (Oegema et al., 2000). Human anillin can bind to actin through a domain located in the N-terminus (Fig. 2; Table 1). *Xenopus* anillin binds to phosphorylated myosin II in vitro via a region that is located just upstream of the actin-binding domain and is conserved in other metazoans (Straight et al., 2005). Consistent with these data, myosin proteins have been identified as binding partners of Anillin in *Drosophila* (Table 1) (D'Avino et al., 2008), but there is no experimental evidence that the myosin-binding domain can bind to myosin in species other than *Xenopus*.
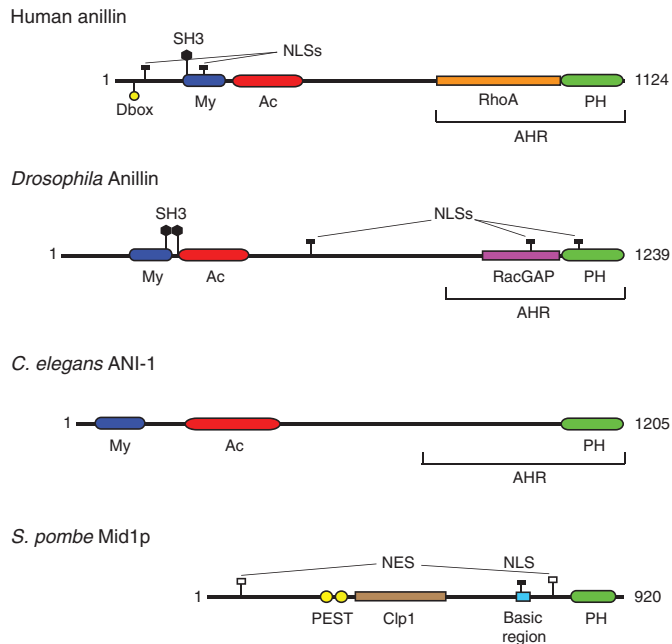
Fig. 2. Secondary structures of Anillin-related proteins in different species. Only ANI-1 and Mid1p are shown for *C. elegans* and *S. pombe*, respectively. Characterized and putative domains are shown. Ac, actin-binding domain; AHR, Anillin homology region; Clp1, region necessary for binding to the Cdc14 homologue Clp1/Flp1; Dbox, destruction box; My, myosin-II-binding domain; NES, nuclear export signal; NLS, nuclear localization signal; PH, pleckstrin-homology domain; RacGAP, region that interacts with the RacGAP50C component of the centralspindlin complex; RhoA, region that interacts with the GTPase RhoA; SH3, Src-homology-3-binding consensus sequences. The two putative NLSs nearest the N-terminus in human anillin are situated very close to each other, and are therefore marked by a single symbol.

Two proteins related to *Drosophila* Anillin, Mid1p and Mid2p, have been identified in fission yeast. Mid1p contains the following regions: a PH domain in the C-terminus, a basic region that includes an NLS, two nuclear export signals (NESs) and two PEST regions, which might mediate its degradation (Fig. 2) (Paoletti and Chang, 2000; Sohrmann et al., 1996). Mid2p was identified by its similarity to both Mid1p and human anillin but, besides the presence of a C-terminal PH motif, no other domains have been described in this protein (Berlin et al., 2003; Tasto et al., 2003).

In conclusion, the general structure of Anillin-related proteins has been relatively well conserved in metazoans: the N-terminal regions contain myosin- and actin-binding domains, and the AHR and PH motifs reside in the C-terminal parts of the proteins (Fig. 2). The *S. pombe* proteins, however, show weaker similarity to their animal counterparts and a strong similarity can only be found in the PH domain (Berlin et al., 2003; Tasto et al., 2003). Unfortunately, although Mid1p is known to interact with myosin II (Motegi et al., 2004), the binding region in Mid1p that is necessary for this interaction is unknown. It would be interesting to discover whether this domain is located in the N-terminal region, as it is in metazoans. In addition, the two Mid proteins colocalize with F-actin but it has never been shown whether they can interact with actin in vivo or in vitro. Should the myosin- and actin-binding domains map to the N-terminal region of Mid1p or Mid2p, this would certainly strengthen the apparent similarity of fungal and animal Anillin-related proteins.

Determinants of Anillin localization
Drosophila
Because Anillin was first identified as an F-actin-binding protein in *Drosophila* embryos (Miller et al., 1989), for historical reasons I will first describe what is known about the localization of the fly protein. *Drosophila* Anillin is nuclear in interphase and relocates to the cortex after nuclear-envelope breakdown. It then accumulates at the equatorial cortex in anaphase and at the cleavage furrow, together with F-actin and myosin II, during telophase (Fig. 3) (Field and Alberts, 1995). Anillin localizes at the nascent cleavage site ahead of F-actin during male meiotic divisions, making it one of the first markers of the nascent furrow (Giansanti et al., 1999). Localization of Anillin to the cleavage furrow does not require F-actin or myosin II, but rather the activity of RhoA and its regulatory factors Pbl and RacGAP50C (Fig. 1B; Table 1). Myosin II depletion does not prevent Anillin accumulation at the equatorial cortex (Hickson and O'Farrell, 2008; Straight et al., 2005), and treatment with F-actin-depolymerizing drugs such as latrunculin A (Lat-A) blocks the cortical localization of Anillin in metaphase but does not prevent its accumulation in a narrow band at the equatorial cortical region in anaphase. However, in the absence of F-actin, Anillin no longer forms a continuous band but instead displays a patchy and/or filamentous distribution; Lat-A-induced Anillin filaments contain septins and myosin II and in some cases colocalize with the plus-ends of the spindle microtubules that contact the cortex (Fig. 1) (D'Avino et al., 2008; Hickson and O'Farrell, 2008). Depletion of RhoA or the RhoGEF Pbl causes Anillin to retain a metaphase-like distribution around the entire cell cortex after anaphase onset (Hickson and O'Farrell, 2008; Somma et al., 2002). When RacGAP50C is down-regulated, Anillin is excluded from one or both cortical polar regions, but fails to accumulate at the nascent cleavage site (D'Avino et al., 2008; Somma et al., 2002). Moreover, Lat-A-induced Anillin filaments no longer colocalize with microtubule plus-ends after RacGAP50C depletion (D'Avino et al., 2008).

The identification of RacGAP50C as a binding partner of Anillin in cultured cells (Table 1) provided further insights into the molecular mechanism involved in recruitment of Anillin to the furrow (D'Avino et al., 2008). Anillin and RacGAP50C were also found to colocalize at the plus-ends of post-anaphase microtubules that contacted the cortex (D'Avino et al., 2008), and their in vivo association was validated by fluorescence resonance energy transfer (FRET) analysis (Gregory et al., 2008). Anillin and RacGAP50C interact directly via a region located in the AHR of Anillin, just upstream of the PH domain (Fig. 2) (D'Avino et al., 2008; Gregory et al., 2008). Consistent with this, the PH domain is unnecessary for proper Anillin accumulation at the furrow, and a fragment including the RacGAP50C binding domain (but lacking the actin- and myosin-binding region and the PH motif) shows weak equatorial cortical localization in telophase and colocalizes with RacGAP50C on the spindle midzone (D'Avino et al., 2008).

The data described above indicate that at least two distinct mechanisms cooperate to localize Anillin at the furrow after anaphase onset. The first, which is mediated by Rho and Pbl but is independent of RacGAP50C, excludes Anillin from the polar regions through a currently unknown molecular mechanism. The recent finding that Anillin binds to RhoA in human cells (Table 1) suggests a possible mechanism for this initial event (Piekny and Glotzer, 2008). The second mechanism involves a direct interaction between Anillin and RacGAP50C and serves to restrict and maintain the localization of Anillin at the cleavage site and

Table 1. Interactors and regulators of Anillin-related proteins

Organism	Protein	Interaction (direct or indirect)	Role of the interaction or regulation	References
Drosophila	F-actin	Yes	CR organization and Anillin localization to the cortex	(Field and Alberts, 1995; D'Avino et al., 2008; Hickson and O'Farrell, 2008)
	Myosin	Yes	CR organization	(D'Avino et al., 2008)
	Pbl	ND	Anillin localization to the CS	(Somma et al., 2002; Hickson and O'Farrell, 2008)
	RacGAP50C	Yes	Anillin localization to the CS	(D'Avino et al., 2008; Gregory et al., 2008)
	RhoA	ND	Anillin localization to the CS	(Somma et al., 2002; Hickson and O'Farrell, 2008)
	Septins	Yes	CR organization	(D'Avino et al., 2008)
Vertebrates	APC/Cdh1	Yes	Anillin degradation	(Zhao and Fang, 2005a)
	ECT2	ND	Anillin localization to the CS	(Zhao and Fang, 2005b)
	F-actin	Yes	CR organization	(Oegema et al., 2000)
	MgcRacGAP	ND	Anillin localization to the CS	(Zhao and Fang, 2005b)
	Myosin	Yes	CR organization	(Straight et al., 2005)
	RhoA	Yes	Anillin localization to the CS	(Piekny and Glotzer, 2008)
	Septins	Yes	CR organization	(Kinoshita et al., 2002)
S. pombe (Mid1p)	Clp1/Flp1	Yes	CR dynamics	(Clifford et al., 2008)
	Myo2p	Yes	CR organization	(Motegi et al., 2004)
	Plo1p	Yes	Mid1p localization	(Bahler et al., 1998)
	Pom1p	ND	Mid1p localization	(Celton-Morizur et al., 2006; Padte et al., 2006)
S. pombe (Mid2p)	F-actin	ND	Mid2p localization	(Berlin et al., 2003; Tasto et al., 2003)
	Septins	ND	Mid2p localization	(Berlin et al., 2003; Tasto et al., 2003)

CR, contractile ring; CS, cleavage site; ND, not determined.

to provide a stable connection between spindle microtubules and the contractile ring during furrow ingression (D'Avino et al., 2008; Gregory et al., 2008). This mechanism might, of course, reinforce the first in a positive-feedback loop because RacGAP50C activates RhoA through its interaction with Pbl (Fig. 1) (Somers and Saint, 2003).

Vertebrates

The human and *Xenopus* homologues of Anillin display a localization pattern that is identical to that of the fly counterpart – they are nuclear in interphase, become cortical after mitotic entry and concentrate at the cleavage furrow in telophase (Oegema et al., 2000; Straight et al., 2005). The N-terminal region of human anillin, which contains the actin- and myosin-binding domains, is necessary for nuclear localization in interphase cells but not for cortical localization in metaphase or cleavage-furrow accumulation in telophase (Oegema et al., 2000). This appears to contrast with the observation that depolymerization of F-actin through Lat-A treatment inhibits recruitment of anillin to the equatorial cortex (Oegema et al., 2000; Straight et al., 2003). The PH domain is essential, though not sufficient, for cortical localization of anillin in metaphase and its robust accumulation at the cleavage site in cytokinesis (Oegema et al., 2000). Moreover, this domain is important for interaction with septins, and their recruitment to the cleavage site (Kinoshita et al., 2002; Oegema et al., 2000). As in *Drosophila*, depletion of RhoA and its activators MgcRacGAP and ECT2 prevents the accumulation of human anillin at the cleavage site (Table 1) (Piekny and Glotzer, 2008; Zhao and Fang, 2005b). Consistent with this, RhoA interacts in vitro with the AHR domain in the C-terminal part of anillin (Piekny and Glotzer, 2008). The entire AHR (including the PH domain) is sufficient to direct localization to the equatorial furrow and is also necessary for RhoA accumulation at the equator (Oegema et al., 2000; Piekny and Glotzer, 2008).

S. pombe

In interphase, Mid1p localizes to the nucleus and, as discrete dots, over a broad region in the medial cell cortex (Bahler et al., 1998;

Paoletti and Chang, 2000; Sohrmann et al., 1996). The medial cortical localization of Mid1p in interphase cells depends on the position of the nucleus (Paoletti and Chang, 2000; Sohrmann et al., 1996). After mitotic entry, Mid1p is exported from the nucleus and its distribution changes from a punctate medial broad band into a compact continuous ring during anaphase; Mid1p then re-enters the nucleus after septum formation (Fig. 3). Mid1p shuttles in and out of the nucleus through the action of its two NESs and a single predominant NLS (Fig. 2). Thus, Mid1p appears to mark the equatorial region in interphase, much earlier than any other components of the contractile ring.

Cortical localization of Mid1p depends on two independent aspects of its secondary structure. First, an amphipathic helix located in the basic region at the C-terminus, just upstream of the NLS, is necessary and sufficient to direct cortical localization. Second, an N-terminal fragment lacking the basic region and the PH domain also exhibits a faint cortical localization and can organize into a tight ring, possibly because of its ability to interact with other ring components (Celton-Morizur et al., 2004; Paoletti and Chang, 2000). The PH domain is not essential for either the localization or function of Mid1p.

During anaphase, Mid1p colocalizes with F-actin and myosin at the contractile ring and interacts with the C-terminal tail of the myosin heavy chain Myo2p (Table 1) (Motegi et al., 2004; Sohrmann et al., 1996). This interaction requires dephosphorylation of Myo2p and is necessary for the initial accumulation of Myo2p to the medial region (Motegi et al., 2004). It is not known whether Mid1p interacts with F-actin as its *Drosophila* counterpart does, but several studies have led to a model in which the cortical nodes of Mid1p promote recruitment of Myo2p, its associated myosin regulatory light chain (MRLC) and the other components of the contractile ring. These nodes then coalesce into a compact contractile ring that drives furrow ingression (Motegi et al., 2004; Paoletti and Chang, 2000; Wu et al., 2003; Wu et al., 2006). Thus, Mid1p is a master organizer of the ring and its localization to the medial cortical region is essential to mark the division site.

Two distinct signalling pathways control Mid1p localization at the medial region of the cell. The first pathway involves the Polo
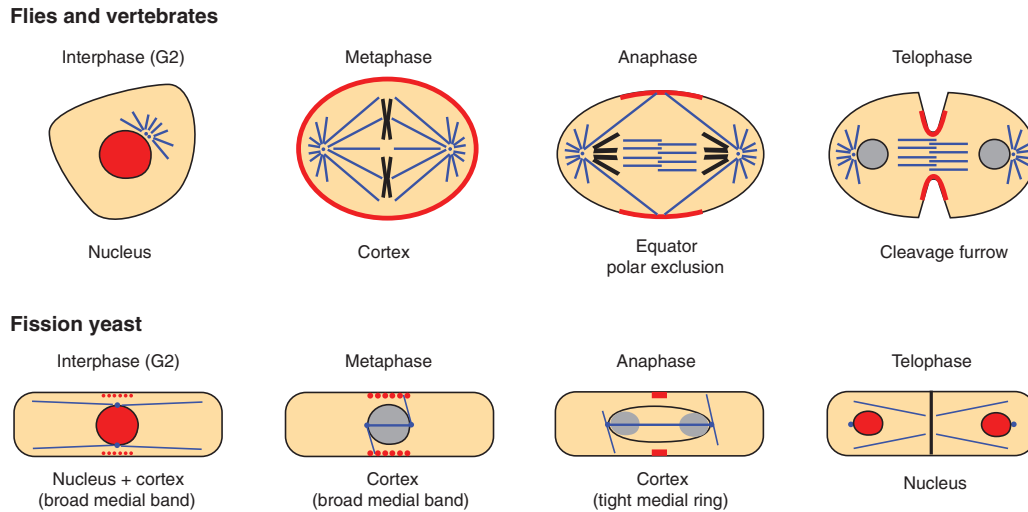
Fig. 3. Localization of Anillin-related proteins during the cell cycle in fission yeast, *Drosophila* and vertebrates. The distribution of Anillin-related proteins is depicted in red, nuclei in grey, chromosomes in black, and microtubules, centrosomes and spindle-pole bodies in blue. ANI-1 also accumulates at the cleavage furrows in *C. elegans* embryos, but its distribution in cycling cells has not been described.

kinase homologue Plo1p (Ohkura et al., 1995), which acts as a positive cue because it promotes Mid1p nuclear export at mitotic entry, increasing its concentration at the equatorial cortex (Bahler et al., 1998). *plo1* and *mid1* interact genetically and physically (Table 1) and Mid1p is phosphorylated during mitosis, but it is unclear whether Plo1p directly phosphorylates Mid1p (Bahler et al., 1998; Sohrmann et al., 1996). Computer simulations indicate that nuclear export per se cannot explain the localization of Mid1p to the medial region; instead, inhibitory mechanisms must also prevent it spreading towards the cell ends (Padte et al., 2006). This second inhibitory pathway involves Pom1p, a DYRK-family kinase that controls polarized cell growth in fission yeast (Bahler and Pringle, 1998). In *pom1* mutants, Mid1p cortical distribution spreads from the medial region to the non-growing end; consequently, the division site also shifts towards the same non-growing tip (Celton-Morizur et al., 2006; Padte et al., 2006). Thus, Pom1p excludes Mid1p from the non-growing end and cooperates with the Plo1p-dependent export of Mid1p from the nucleus to localize Mid1p in a medial cortical band. Another, as-yet-unidentified signalling pathway must also prevent Mid1p from associating with the growing end.

Function of Anillin-related proteins
Drosophila
The function of Anillin in *Drosophila* has been elucidated by the study of *anillin* (also known as *scraps*) mutants and by RNAi-dependent Anillin depletion in both cultured cells and flies. The actomyosin ring is assembled normally in early telophase cells after *anillin* RNAi, but it becomes severely disorganized in late telophase, exhibiting aberrant F-actin accumulation and the formation of numerous membrane blebs; this ultimately leads to a late failure of cytokinesis (Echard et al., 2004; Somma et al., 2002). Thus, Anillin is not required for F-actin accumulation at the furrow but rather for proper organization of the contractile ring. Similarly, recruitment of myosin II to the cleavage site does not require Anillin (Straight et al., 2005), although a recent time-lapse study reported abnormal furrow oscillations along the spindle axis in Anillin-depleted cells, which was followed by mislocalization of the MRLC (Hickson and

O'Farrell, 2008). However, this unstable-furrow phenotype was not observed in previous studies that used fixed preparations (Somma et al., 2002; Straight et al., 2005) or in time-lapse analysis using a similar cell line expressing a tubulin-GFP-encoding transgene (Echard et al., 2004); the reasons for this discrepancy are unclear.

Analysis of *anillin* mutant animals demonstrated that Anillin is essential for viability and functions in cytokinesis, for pole-cell formation and for cellularization in *Drosophila* embryos. Indeed, Anillin concentrates at the leading edge of the membrane invaginations that are responsible for cellularization during early embryogenesis, which are enriched in both F-actin and myosin II. Furthermore, the localization of myosin II and septins is disrupted in *anillin* mutant embryos (Field et al., 2005; Thomas and Wieschaus, 2004). Anillin is also essential for the recruitment of the septins Peanut and Septin 2 to the furrow in cultured *Drosophila* cells. These proteins often mislocalize on central-spindle microtubules following *anillin* RNAi, which suggests that septins travel along spindle microtubules to reach the cortex where they interact with Anillin (D'Avino et al., 2008). By contrast, septins are not required for proper Anillin localization at the cleavage furrow in cultured cells or embryos (Adam et al., 2000; D'Avino et al., 2008). Finally, although Anillin is not required for RacGAP50C localization to the spindle midzone (D'Avino et al., 2008; Gregory et al., 2008), it does seem to be necessary for RacGAP50C accumulation at the cortex in larval brain cells (Gregory et al., 2008). Altogether, these data indicate that Anillin is essential for the proper organization of actomyosin contractile structures, but not their initial assembly or contractile activity, during both cytokinesis and embryonic cellularization.

C. elegans
Of the three Anillin-related proteins in *C. elegans* (ANI-1, ANI-2 and ANI-3), only ANI-1 appears to be important for contractile-ring formation (Maddox et al., 2005). RNAi of *ani-3* does not lead to any abnormalities, either alone or in combination with depletion of ANI-1 or ANI-2. ANI-2 localizes to the hermaphrodite gonad and is required for the structural organization of this organ. *ani-1* knockdown causes a series of defects in actomyosin-mediated contractile

processes in early embryos, which include polar-body formation, membrane ruffling and pseudo-cleavage (Maddox et al., 2005). In addition, the cleavage furrow of the first embryonic division, which usually ingresses asymmetrically, becomes symmetric in *ani-1* RNAi embryos (Maddox et al., 2007). Consistent with these phenotypes, ANI-1 concentrates in cortical patches during membrane ruffling and pseudo-cleavage, and at the cleavage furrow and contractile rings during the first embryonic cytokinesis and polar-body extrusion (Maddox et al., 2005). As with *Drosophila* Anillin, ANI-1 is necessary for the recruitment of septins to the contractile rings and, accordingly, loss of asymmetric furrowing is observed in septin-depleted embryos (Maddox et al., 2005; Maddox et al., 2007). ANI-1 is not required for accumulation of myosin II to the cleavage furrow, and vice versa. By contrast, both myosin II and septins fail to organize into cortical patches before the first embryonic mitosis in *ani-1* RNAi embryos, and this most probably accounts for the absence of membrane ruffling and pseudo-cleavage (Maddox et al., 2005).

In contrast to Anillin-related proteins in flies and vertebrates, ANI-1 is not essential for cytokinesis in *C. elegans*. However, the fact that some multinucleate cells are present during later development in *ani-1* RNAi embryos indicates that cytokinesis is less robust in the absence of ANI-1 (Maddox et al., 2007). In addition, double knockdown of *ani-1* and other factors involved in cytokinesis, such as ROK and ZEN-4, increases the rate and severity of cytokinesis failure (Maddox et al., 2007; Werner and Glotzer, 2008). On the basis of their results, Maddox et al. proposed that furrow asymmetry makes the cytokinesis process robust in the face of mechanical changes (Maddox et al., 2007).

Human cells

During the cell cycle in human cells, levels of anillin are regulated through ubiquitin-dependent degradation, which is mediated by the anaphase-promoting complex (APC; an E3 ubiquitin ligase) and its activating factor Cdh1 (Table 1); Cdh1 associates with a destruction-box (D-box) motif in the N-terminal region of anillin (Fig. 2) (Zhao and Fang, 2005a).

Perturbing anillin function in human tissue culture cells, either by antibody microinjection or RNAi-mediated silencing, causes severe cytokinesis defects (Oegema et al., 2000; Straight et al., 2005; Zhao and Fang, 2005a). The early stages of furrow formation and ingression are generally unaffected after RNAi, but the furrow becomes unstable and oscillates along the spindle axis, in concert with abnormal cortical contractions that require actin and myosin activity (Straight et al., 2005; Zhao and Fang, 2005a). Consistent with these observations, myosin II and MRLC accumulate normally at the cleavage furrow in early telophase but then become mislocalized along the cortex following the oscillations of the furrow (Straight et al., 2005; Zhao and Fang, 2005a). Thus, although anillin is not required for the initial furrowing events or the accumulation of myosin at the cleavage site, it is necessary to maintain both furrow position and myosin localization during ingression.

Immunostaining experiments have indicated that RhoA does not accumulate at the cleavage site following *anillin* RNAi (Piekny and Glotzer, 2008; Zhao and Fang, 2005a). However, overexpressed transgenic GFP-RhoA is still detected at the furrow and does not move extensively during the furrow oscillations in *anillin* RNAi cells (Piekny and Glotzer, 2008). Although the distribution of the overexpressed GFP-RhoA might not exactly reflect that of the endogenous protein, these data indicate that anillin is required for RhoA accumulation at the furrow but not for its initial recruitment.

Depletion of the MgcRacGAP component of the centralspindlin complex affects anillin accumulation at the furrow, but *anillin* RNAi does not affect MgcRacGAP localization (Zhao and Fang, 2005a; Zhao and Fang, 2005b). However, knockdown of anillin or the centralspindlin-complex components impairs accumulation of RhoA at the equatorial cortex, although not in identical ways (Kamijo et al., 2006; Nishimura and Yonemura, 2006; Piekny and Glotzer, 2008; Yuce et al., 2005; Zhao and Fang, 2005a; Zhao and Fang, 2005b). Simultaneous depletion of both MKLP1 and anillin causes more severe phenotypes than either knockdown alone. In these double-depleted cells, cleavage furrows never form and RhoA, actin and myosin are mislocalized around the entire cortex. These results have been interpreted to indicate that centralspindlin and anillin can independently promote RhoA localization at the furrow (Piekny and Glotzer, 2008). However, anillin interacts via its conserved AHR with the RacGAP50C component of the centralspindlin complex in *Drosophila* (D'Avino et al., 2008; Gregory et al., 2008); if the same interaction occurs in humans, the above interpretation should be revisited as these proteins might act collaboratively, rather than independently, to promote RhoA localization and activation at the cleavage site (Fig. 4).

S. pombe

Mid1p and Mid2p participate in distinct processes during cytokinesis. The *mid1* gene (also known as *pos1* and *dmf1*) was identified in screens for mutants that affect the position of the contractile ring and septum (Chang et al., 1996; Edamatsu and Toyoshima, 1996). In *mid1* mutants, the actomyosin contractile ring and septum are assembled normally but are positioned at random locations and angles on the cell cortex, even though the nucleus is correctly located in the middle of the cell (Chang et al., 1996; Sohrmann et al., 1996). Thus, Mid1p and the formation of cortical nodes are not essential for the assembly of the contractile ring, but only for its correct position (Huang et al., 2008). A recent study indicates that, in the absence of Mid1p, the septation initiation network (SIN), which is responsible for the temporal coordination of cytokinesis, promotes contractile-ring assembly through the formation of actomyosin filaments, rather than through a cortical network of proteins (Hachet and Simanis, 2008). These data indicate that Mid1p and the SIN function together to orchestrate the assembly of the contractile ring in fission yeast.

Although the microtubule cytoskeleton appears normal throughout the cell cycle in *mid1* mutants (Chang et al., 1996; Sohrmann et al., 1996), *mid1* is necessary for proper spindle alignment during mitosis (Gachet et al., 2004). As the rotation of the mitotic spindle depends on the interaction of astral microtubules with the medial cortex and the contractile ring, these results suggest that Mid1p might mediate this interaction. Recent evidence indicates that Mid1p also helps to maximize the signalling efficiency of the Cdc14 phosphatase homologue Clp1/Flp1 (Clifford et al., 2008). Clp1 interacts directly with Mid1p (Fig. 2) and this association is responsible for its recruitment to the contractile ring, where it dephosphorylates contractile-ring components such as Cdc15p, influencing their dynamics. Thus, Mid1p-mediated recruitment of Clp1 might be essential to confer necessary plasticity on the contractile ring, possibly by antagonizing the activity of kinases such as Plo1p.

The other Anillin-related protein in *S. pombe*, Mid2p, was identified by its sequence similarity to Mid1p (Berlin et al., 2003; Tasto et al., 2003). Levels of Mid2p peak at septation and are regulated by ubiquitin-mediated proteolysis (Tasto et al., 2003). In *mid2* mutants, the contractile ring is formed and positioned normally,
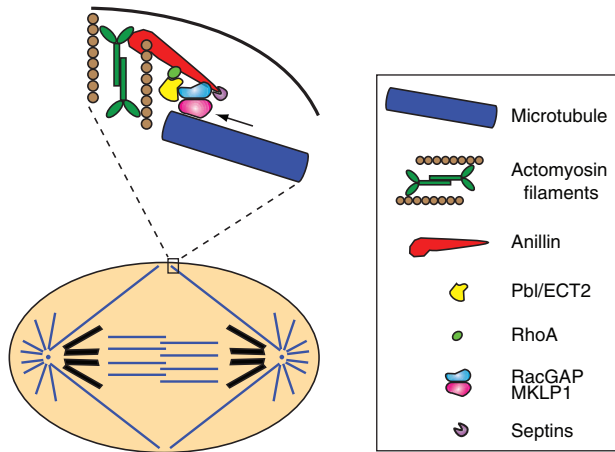
Fig. 4. A model for Anillin functions in metazoans. Interaction between Anillin and the RacGAP component of the centralspindlin complex connects the actomyosin filaments in the contractile ring with the spindle microtubules. Anillin also functions as a scaffold protein to maximize the efficiency of cleavage furrow ingression by bringing together the signalling and structural components necessary for the assembly and contraction of the actomyosin contractile ring.

but cell separation is delayed (Berlin et al., 2003; Tasto et al., 2003). Consistent with these different phenotypes, the two *mid* genes do not share overlapping functions (Berlin et al., 2003). Mid2p organizes into a medial ring in late anaphase that then splits into two separate rings during septation, mirroring the distribution of septins. Consistently, Mid2p is required for the organization and stabilization of the septin ring. Conversely, Mid2p localization depends on septins and F-actin. Finally, unlike Mid1p, the PH domain of Mid2p is necessary, albeit not sufficient, for proper localization and function (Berlin et al., 2003; Tasto et al., 2003). It is not yet known, however, whether this domain interacts with septins as it does in metazoans.

Common and distinct features of Anillin-related proteins
Protein structure
Although the PH domain is very well conserved in all Anillin-related proteins, its function differs between species. The PH domain is necessary for proper function of anillin and robust cleavage-furrow localization in human cells (Oegema et al., 2000; Piekny and Glotzer, 2008), but it is totally dispensable for furrow localization in flies (D'Avino et al., 2008). Nonetheless, mutations in the PH domain of *Drosophila* Anillin impair its function and prevent septin recruitment to the cellularization front and cleavage furrow (Field et al., 2005), suggesting that the PH domain might mediate the interaction with septins as it does in vertebrates (Kinoshita et al., 2002). In fission yeast, the PH domain is not required for localization and function of Mid1p (Paoletti and Chang, 2000), whereas it is essential for activity and distribution of Mid2p (Berlin et al., 2003; Tasto et al., 2003). Although the localizations of septins are altered in *mid2* mutants, it is still unclear whether the PH domain of Mid2p interacts with septins.

The region in the AHR upstream of the PH domain displays a high degree of sequence similarity in all metazoans and is essential for the proper localization and function of Anillin in both fly and human cells (D'Avino et al., 2008; Oegema et al., 2000; Piekny and Glotzer, 2008). Moreover, this region harbours binding sites for RhoA in humans (Piekny and Glotzer, 2008) and RacGAP50C

in flies (D'Avino et al., 2008; Gregory et al., 2008), suggesting that the interaction of Anillin with these two proteins is conserved in all metazoans. Interestingly, an amphipathic helix located upstream of the PH domain is also necessary for Mid1p cortical localization in fission yeast (Celton-Morizur et al., 2004). This indicates that the C-terminal region of Anillin, either including or excluding the PH domain, has a crucial role for the function and localization of these proteins in both fungi and metazoans.

Subcellular localization
The localization of Anillin-related proteins in diverse organisms is strikingly similar (see above). They all localize to the equatorial cortical region from late anaphase and throughout telophase and, with the exception of ANI-1 in worms and Mid2p in fission yeast, they accumulate in the nucleus in interphase (Berlin et al., 2003; Field and Alberts, 1995; Maddox et al., 2005; Oegema et al., 2000; Paoletti and Chang, 2000; Tasto et al., 2003). In the majority of cases, early during metaphase, Anillin-related proteins accumulate in nodes or puncta that then coalesce into a continuous ring at the furrow. Perhaps the only difference has been seen in *Drosophila*, in which Anillin does not form puncta, but is instead uniformly distributed all around the cortex in metaphase (Field and Alberts, 1995). However, this uniform distribution in flies clearly depends on the strong affinity of Anillin for F-actin (D'Avino et al., 2008; Hickson and O'Farrell, 2008), and thus it seems more likely to be the result of a passive, rather than an active, recruitment mechanism. Consistent with these results, the myosin- and actin-binding domains are necessary for cortical localization in flies but not in humans (D'Avino et al., 2008; Oegema et al., 2000; Piekny and Glotzer, 2008), and a fragment of *Drosophila* Anillin that contains only these two domains colocalizes with F-actin throughout the cell cycle (P.P.D., unpublished observations).

In both fission yeast and flies, ANI-1 is one of the first factors to localize to the future division site (Giansanti et al., 1999; Paoletti and Chang, 2000), making it one of the earliest markers of the nascent cleavage furrow. However, the two opposing mechanisms that exclude Anillin from the polar regions and promote its accumulation to the equatorial cortex differ between fungi and animals. In fission yeast, the Pom1p kinase and an unidentified factor prevent Mid1p from spreading to the cell ends, whereas the Polo kinase homologue Plo1p increases Mid1p concentration to the medial region by promoting its nuclear export at mitotic entry (see above) (Bahler et al., 1998; Celton-Morizur et al., 2006; Padte et al., 2006). In *Drosophila*, and possibly in all metazoans, a RhoA-mediated pathway is responsible for the exclusion of Anillin from the polar regions in anaphase, whereas an interaction with the RacGAP50C component of the centralspindlin complex maintains and restricts its localization at the cleavage furrow during telophase (D'Avino et al., 2008; Gregory et al., 2008; Hickson and O'Farrell, 2008). It should also be noted that astral microtubules suppress RhoA activation at the polar cortex in mammalian cells (Murthy and Wadsworth, 2008). Thus, it appears that the opposing signals that promote Anillin localization at the equatorial cortex in animals are generated by two distinct parts of the spindle – astral microtubules (which mediate RhoA inhibition) and central-spindle microtubules (which promote the interaction of Anillin with the RacGAP component of the centralspindlin complex).

New roles for Anillin-related proteins
The principal function that is shared by all Anillin-related proteins is to associate with the major structural components of the

actomyosin ring and to provide a scaffold for its proper organization. Out of all of the Anillin-related proteins, only *S. pombe* Mid1p is also involved in positioning the cleavage plane. However, recent studies have reported two additional functions for Anillin-related proteins. First, this scaffold protein also brings together the signalling factors that regulate contractile-ring dynamics, including RhoA, its RhoGEF activator Pbl/ECT2 and the RacGAP component of the centralspindlin complex in metazoans (Fig. 4) (D'Avino et al., 2008; Gregory et al., 2008; Piekny and Glotzer, 2008), and the Cdc14 homologue Clp1/Flp1 in fission yeast (Clifford et al., 2008). Therefore, by linking signalling proteins with structural components, Anillin proteins help to maximize the efficiency of the reactions that regulate actomyosin-ring dynamics and cleavage-furrow ingression. Second, the interaction between Anillin and the microtubule-bound centralspindlin complex connects the actomyosin ring with spindle microtubules at the cleavage site in *Drosophila* cells (D'Avino et al., 2008; Gregory et al., 2008). Although it is not yet known whether a similar mechanism exists in other organisms, astral microtubules interact with the medial cortex to align the spindle in fission yeast and this process is impaired in *mid1* mutants (Gachet et al., 2004).

Evolution of Anillin-related proteins

Did fungal and metazoan Anillin proteins evolve from a common ancestor or are they the result of convergent evolution? Unfortunately there is no conclusive evidence to decisively support either hypothesis. Interspecies functional-rescue experiments have not been reported and even these might not answer this question. Some of the basic structural features of Anillin, such as the C-terminal PH domain, are clearly present in both fungi and metazoans (Fig. 2). Assuming that the common-ancestor hypothesis is correct, I would like to speculate that the *S. pombe mid1* and *mid2* genes are the result of a duplication event, because the two proteins have complementary functions that are carried out by a single protein in metazoans. For instance, Mid1p binds to myosin and Mid2p interacts with septins, whereas in animal cells a single anillin protein is able to interact with both types of proteins (Table 1).

Is the role of Mid1p in positioning the cleavage site in fission yeast the result of specialized evolution or a function that has been lost in metazoans? My preference is for the latter. Probably, Anillin originally had a role in determining the division plane in unicellular organisms, in which this plane needs to be linked to the position of the genomic material. In metazoans, the orientation of cell division must also account for the future location of the two daughter cells in tissues. This is especially important in specialized mitoses such as the asymmetric divisions of stem cells. Thus, it is possible that, in multicellular organisms, the cell cytoskeleton – and in particular the mitotic spindle – has acquired the ability to signal the position of the cleavage plane after the segregation of the genomic material. In such a scenario, the original role of Anillin in determining the division site would be lost.

Conclusions and future perspectives

The studies described in this article indicate that Anillin-related proteins have key roles in organizing the contractile ring during cytokinesis. However, it is important to point out that these proteins are not essential for the assembly and contraction of the actomyosin filaments. Indeed, in animal cells deprived of Anillins by either mutation or RNAi, the cleavage furrow is always able to ingress, either in part or completely, and cytokinesis fails at quite a late stage (Field et al., 2005; Somma et al., 2002; Straight et al., 2005;

Zhao and Fang, 2005a). Moreover, in *S. pombe*, the two *mid* genes are not necessary for viability (Berlin et al., 2003; Chang et al., 1996; Sohrmann et al., 1996; Tasto et al., 2003).

Despite the large body of data that exists regarding the functions of Anillin-related proteins in cytokinesis, there are still several unanswered questions. First, is the interaction of Anillin-related proteins with their multiple partners regulated by post-translational events, such as phosphorylation or dephosphorylation? If so, which kinases and phosphatases are responsible for these modifications? The Polo kinase homologue Plo1p and the Ccd14 homologue Clp1/Flp1 are two potential candidates in fission yeast, but there is no evidence that any mitotic kinase or phosphatase regulates Anillin functions in metazoans. Second, does the presence of a PH domain indicate that Anillin mediates the interaction of the contractile ring with the plasma membrane, as was originally hypothesized (Oegema et al., 2000)? No conclusive evidence has yet been found to support such a role for Anillin in any organism, even though Mid1p has been found to associate with membranes via a C-terminal amphipathic helix (Celton-Morizur et al., 2004). The recent finding that Inn1, a protein containing a C2 lipid-binding domain, links the invaginating membrane to the actomyosin ring in the yeast *Saccharomyces cerevisiae* (Sanchez-Diaz et al., 2008) highlights the possibility that other proteins might also be important for establishing a connection between the membrane and the contractile ring at the cleavage site. Thus, a careful analysis of membrane formation and ingression in cells deprived of Anillin is needed to definitively prove whether Anillin links the contractile ring to the plasma membrane. Furthermore, even if Anillin is not required for coupling membrane invagination to actomyosin-ring contraction, it is still possible that this protein functions as a docking platform for the vesicles that are transported along the central-spindle microtubules and that ultimately merge with the furrowing membrane during cytokinesis (Prekeris and Gould, 2008).

In conclusion, the studies summarized here, which span 20 years from the initial discovery of Anillin in *Drosophila* (Miller et al., 1989), have clearly established that Anillin-related proteins have a key role in the organization of the contractile ring and contribute to the efficiency of the signalling pathways that control ring dynamics in many organisms. Nonetheless, the functional repertoire of this family of cytokinesis proteins has not yet been fully unveiled and their analysis will keep the researchers in the field of cytokinesis busy for some years to come. Finally, the findings that Anillin is overexpressed in diverse human tumours and has a key role in lung carcinogenesis (Hall et al., 2005; Suzuki et al., 2005) make this protein an interesting potential target for anti-cancer therapies.

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