SPIN90 associates with mDia1 and the Arp2/3 complex to regulate cortical actin organization

Luyan Cao¹,¹⁵, Amina Yonis²,³,¹⁵, Malti Vaghela²,⁴,¹⁵, Elias H. Barriga³,¹², Priyamvada Chugh⁵, Matthew B. Smith⁵,¹³, Julien Maufort⁶,⁷, Geneviève Lavoie⁸, Antoine Méant⁸, Emma Ferber², Miia Bovellan²,⁵, Art Alberts⁹,¹⁷, Aurélie Bertin⁶,⁷, Roberto Mayor⁶,⁷, Ewa K. Paluch⁵,¹⁰,¹⁴, Philippe P. Roux⁸,¹¹, Antoine Jégou¹⁵,¹⁶ ✉, Guillaume Romet-Lemonne¹⁵,¹⁶ ✉ and Guillaume Charras¹⁵,²,³,¹⁰,¹⁶ ✉

Cell shape is controlled by the submembranous cortex, an actomyosin network mainly generated by two actin nucleators: the Arp2/3 complex and the formin mDia1. Changes in relative nucleator activity may alter cortical organization, mechanics and cell shape. Here we investigate how nucleation-promoting factors mediate interactions between nucleators. In vitro, the nucleation-promoting factor SPIN90 promotes formation of unbranched filaments by Arp2/3, a process thought to provide the initial filament for generation of dendritic networks. Paradoxically, in cells, SPIN90 appears to favour a formin-dominated cortex. Our in vitro experiments reveal that this feature stems mainly from two mechanisms: efficient recruitment of mDia1 to SPIN90–Arp2/3 nucleated filaments and formation of a ternary SPIN90–Arp2/3–mDia1 complex that greatly enhances filament nucleation. Both mechanisms yield rapidly elongating filaments with mDia1 at their barbed ends and SPIN90–Arp2/3 at their pointed ends. Thus, in networks, SPIN90 lowers branching densities and increases the proportion of long filaments elongated by mDia1.

Living cells have a remarkable ability to change shape during physiological processes such as division, migration and differentiation. These shape changes are governed by mechanical changes in the cortex, a thin network of actomyosin below the membrane. Changes in cortical mechanics can originate from changes in myosin activity or cortex architecture, which arise from changes in actin filament length or network organization. One potential mechanism to control cortex architecture involves regulation of actin nucleators. Indeed, in vitro—in the presence of profilin—formins generate longer filaments than those created by Arp2/3-mediated branching and in cells, single-molecule experiments suggest a similar trend. Furthermore, actin nucleators generate varied network topologies, ranging from highly branched networks generated by the Arp2/3 complex to linear arrays generated by formins and Ena/VASP proteins. Thus, a switch in the dominant F-actin nucleator might also alter network organization. However, little is known about how nucleator activity is controlled to change network topology and mechanics.

Many cellular actin-based structures such as the cortex, the leading edge of migrating cells, phagocytic cups and intercellular junctions require both formins and the Arp2/3 complex for their formation. This is surprising, because the actin networks they generate differ extensively in their topology, protein interactors, dynamics and force generation. Some reports have shown synergistic action of pointed end nucleators (such as Spire and adenomatous polyposis coli) with barbed end nucleators (such as formins), whereas others have shown sequential action of nucleators. Overall, these observations underscore the importance of nucleator crosstalk for the generation of functionally optimal actin structures in cells.

In addition to RhoGTPases, nucleation-promoting factors (NPFs) are involved in activating nucleators or maintaining their activity. The best studied NPF is probably the wave regulatory complex (WRC), which consists of five subunits (SRA1, NAP1, ABI1, BRK1 and WAVE2) and activates the Arp2/3 complex to generate branched actin networks. After the WAVE complex detaches from Arp2/3, another NPF, cortactin, protects Arp2/3 against debranching. Some NPFs can interact with multiple nucleators, making them prime candidates as mediators of interplay. IQGAP1 can maintain the activity of mDia1 via its C-terminal Dia1-binding region (DBR) and promote Arp2/3 activity by interacting with N-WASP and the WAVE complex via its N-terminal calponin homology domain. Another NPF, SPIN90 (also known as DIP, WISH or NCKIPSD), has been reported to interact with Arp2/3 in some studies and with formins in others. SPIN90 forms a complex with Arp2/3 to stimulate formation of unbranched filaments.

¹Université de Paris, CNRS, Institut Jacques Monod, Paris, France. ²London Centre for Nanotechnology, University College London, London, UK. ³Department of Cell and Developmental Biology, University College London, London, UK. ⁴Department of Physics and Astronomy, University College London, London, UK. ⁵MRC Laboratory for Molecular Cell Biology, University College London, London, UK. ⁶Laboratoire Physico Chimie Curie, Institut Curie, PSL Research University, CNRS UMR168, Paris, France. ⁷Sorbonne Universités, Paris, France. ⁸Institute for Research in Immunology and Cancer, Université de Montréal, Montréal, Quebec, Canada. ⁹Van Andel research institute, Grand Rapids, MI, USA. ¹⁰Institute for the Physics of Living Systems, University College London, London, UK. ¹¹Department of Pathology and Cell Biology, Université de Montréal, Montréal, Canada. ¹²Present address: Instituto Gulbenkian de Ciência, Oeiras, Portugal. ¹³Present address: The Francis Crick institute, London, UK. ¹⁴Present address: Physiology, Development and Neuroscience, University of Cambridge, Cambridge, UK. ¹⁵These authors contributed equally: Luyan Cao, Amina Yonis, Malti Vaghela. ¹⁶These authors jointly supervised this work: Antoine Jégou, Guillaume Romet-Lemonne, Guillaume Charras. ¹⁷Deceased: Art Alberts. ✉e-mail: antoine.jegou@ijm.fr; romet@ijm.fr; g.charras@ucl.ac.uk
In addition to providing the initial filament necessary for generation of dendritic networks by the WRC and Arp2/3\cite{34}, recent work suggests that SPIN90 competes with the WRC to modulate the degree of branching in networks\cite{35}. Furthermore, SPIN90 can interact with the diaphanous related formins (DRE) mDia1 and mDia2 via its leucine-rich repeat (LRR) and/or SRC homology 3 (SH3) domains\cite{36,37} (Fig. 1a) and, surprisingly, it inhibits actin filament elongation by mDia2 but not mDia1\cite{38}.

In this study, we examine how NPFs regulate nucleator activity in the actin cortex to control its organization, assembly kinetics and mechanics. We show that IQGAP1 controls the activity of the formin mDia1, WRC regulates Arp2/3 branching activity and SPIN90 mediates an unexpected synergistic action between Arp2/3 and mDia1.

**Results**

**Several NPFs localize to the actin cortex.** Previous proteomic analyses revealed the presence of two actin nucleators in the cortex of M2 blebbing melanoma cells, the Arp2/3 complex and the formin mDia1. Several NPFs that could regulate these nucleators were also present (Supplementary Table 1): the WRC, IQGAP1, cortactin, SPIN90 and flightless-I homologue (Fli-I), an NPF that prevents formin autoinhibition\cite{39}. Published proteomic datasets indicate that these NPFs are also expressed in HeLa cells (Supplementary Table 2), suggesting they may have a general role in controlling cortical nucleator activity\cite{40,41,42}.

We examined NPF localization in M2 melanoma cell blebs and metaphase HeLa cells. Blebs provide a snapshot into the cortex life cycle\cite{43}, whereas mitosis represents a key physiological function of the cortex. All NPFs identified in proteomics localized to the cortex of mitotic HeLa cells and retracting blebs, in which a cortex reforms during the mitotic cycle\cite{44} (Fig. 1b,c and Extended Data Fig. 1a–d). Thus, these NPFs are promising candidates for controlling the activity of cortical nucleators.

We concentrated on WRC and SPIN90 because they mediate the transition from branched to unbranched actin networks nucleated by Arp2/3, and IQGAP1 and SPIN90 because they may coordinate the activity of Arp2/3 and mDia1.

**SPIN90 depletion mimics mDia1 depletion in blebbing cells.** To determine how NPFs modulate nucleator activity, we examined their effect on bleb size in M2 cells, knowing that Arp2/3 depletion results in small blebs, whereas mDia1 depletion results in large blebs\cite{45}. As expected, IQGAP1 depletion resulted in more cells with large blebs (Fig. 1d and Supplementary Fig. 1h,i), consistent with a role in maintaining mDia1 activity\cite{46}, and depletion of WRC subunits resulted in small blebs (Fig. 1c,f and Supplementary Fig. 1h,k,l), consistent with its known role in regulating Arp2/3. Surprisingly, SPIN90 depletion resulted in large blebs (Fig. 1d,f and Supplementary Fig. 1h), suggesting that SPIN90 cooperates with mDia1 directly or indirectly rather than inhibiting DRFs as previously reported\cite{47}. These results (summarized in Supplementary Table 3) led us to focus on SPIN90.

**SPIN90 depletion perturbs cell proliferation.** Previous work showed that mDia1 depletion increased cell death, whereas Arp2/3 depletion did not\cite{48}. When we examined how NPF depletion affected proliferation in HeLa cells, we found that depletion of WRC subunits did not increase cell death, similar to Arp2/3 depletion, but IQGAP1 depletion led to a twofold increase in cell death (Fig. 1g,h and Supplementary Fig. 1a–g). This latter effect was probably mediated by interaction with mDia1, because expression of the mouse IQGAP1 DBR domain in IQGAP1-depleted cells decreased cell death to near baseline levels (Fig. 1a,i). Similar to mDia1 depletion, SPIN90 depletion increased cell death threefold (Fig. 1h and Supplementary Fig. 1a–g). We confirmed the specificity of depletion by expressing full-length mouse SPIN90 in SPIN90-depleted cells (Fig. 1i). Examination of changes in mRNA transcript abundance by quantitative PCR confirmed that the effect of SPIN90 and IQGAP1 depletion was not due to indirect regulation of NPFs or nucleators at the transcriptional level (Supplementary Fig. 2). Thus, mDia1 activity regulated by IQGAP1 and SPIN90 is necessary for proliferation (Supplementary Table 3).

**SPIN90 depletion decreases cortical mesh size in blebs.** We then examined how NPF depletion affects the organization of F-actin in the cortex of M2 cell blebs. Previous work showed that depletion of mDia1 led to large gaps in the cortex, whereas Arp2/3 depletion led to no clear change in actin filament density\cite{49}. IQGAP1 depletion led to a twofold increase in the proportion of gaps with diameters larger than 140 nm (Fig. 2a,b and Supplementary Fig. 3), similar to mDia1 depletion. NAP1 depletion did not change gap diameter distribution, similar to ACTR2 depletion (Fig. 2a,b and Supplementary Fig. 3). SPIN90 depletion led to a visibly denser cortex (Fig. 2a) and a 20% increase in the proportion of gaps less than 30 nm in diameter (Fig. 2b and Supplementary Fig. 3). This was unexpected, because previous cell-scale assays showed that SPIN90 depletion phenocopied mDia1 depletion (Fig. 1). However, SPIN90 has been proposed to compete with WRC for Arp2/3 to regulate the degree of branching\cite{50}. Depletion of SPIN90 might therefore lead to a denser cortical network because of increased branching. Thus, SPIN90 may mediate crosstalk between mDia1 and Arp2/3 (Supplementary Table 3).

**SPIN90 governs cortical actin density and thickness in mitotic cells.** We next determined whether NPF depletion affected cortical thickness and density during mitosis in HeLa cells by analysing the fluorescence profile of an F-actin fluorescent reporter with respect to the plasma membrane\cite{51} (Fig. 2c). Previous work showed that depletion of mDia1 leads to a decrease in cortical thickness, whereas depletion of Arp2/3 does not affect thickness\cite{52}. Depletion of IQGAP1 and NAP1 had no impact on cortical thickness or density (Fig. 2d,e). Similar to mDia1 depletion, SPIN90 depletion substantially decreased cortical thickness by about 25% (Fig. 2d). However, SPIN90 depletion also led to an increase of about 50% in cortex density (Fig. 2e), something not observed with mDia1 depletion\cite{53}, but consistent with the smaller cortical gap sizes measured by scanning electron microscopy (SEM) (Fig. 2a,b).

**SPIN90 controls cortical actin accumulation rate in mitotic cells.** To quantitatively examine the role of NPFs in regulating nucleator activity, we measured the cortical F-actin accumulation rate in blebs generated by ablation of the cortex of a metaphase HeLa cell using a pulsed-UV laser\cite{54} (Fig. 3a and Extended Data Fig. 2). Previous work showed that depletion of mDia1 decreases F-actin accumulation rate, whereas depletion of Arp2/3 subunits increases it\cite{55}. Depletion of IQGAP1 and NAP1 mirrored the respective effects of mDia1 and Arp2/3 depletion, consistent with their proposed roles (Fig. 3c,d). SPIN90 depletion led to a notable decrease in actin regrowth rate, providing further evidence for cooperation with mDia1 (Fig. 3b,d). Together, these results indicate that NPFs regulate actin-network growth kinetics in the mitotic cortex (Supplementary Table 3).

**SPIN90 depletion stiffens the mitotic cortex.** Recent work has shown that modulating nucleator activity affects cell mechanics\cite{56,57}. To probe cortical mechanics, we indented rounded metaphase HeLa cells with a blunt atomic force microscopy (AFM) tip, limiting indentation to depths of less than 500 nm; the cortex dominates mechanics in this range\cite{58} (Fig. 3e). The apparent stiffness measured is sensitive to contributions from cortical elasticity and tension.

Inhibition of formins with SMIFH2 led to a notable decrease in apparent stiffness, consistent with previous results\cite{59} (Fig. 3g). However, mDia1 depletion had no effect (Fig. 3g), perhaps because of a more acute effect of SMIFH2 or a low sensitivity of apparent
**Fig. 1** Several NPFs are present in the actin cortex. **a**, Domain structure of IQGAP1, mDia1 and SPIN90. Amino acid position is given from the N terminus. Known protein interactors are shown below the domains. Known positive and inhibitory interactions between IQGAP1, mDia1 and SPIN90 are indicated. CHD, calponin homology domain; FH1 and FH2, formin homology domains; DBR, Diaphanous autoregulatory domain; PRD, proline rich domain; GBD, GTPase binding domain; GRD: RasGAP-related domain; RGCT, RasGAP C-terminal domain; LRR, leucine rich region; WWIQ, WW domain and isoleucine and glutamine (IQ) motifs. **b**, Localization of nucleators and NPFs in M2 blebbing melanoma cells. **c**, IQGAP1 localization was determined by transient transfection with IQGAP1–green fluorescent protein (GFP). The SRA1 subunit of the WAVE complex and SPIN90 were detected by immunofluorescence. White arrowheads indicate expanding blebs and red arrowheads indicate retracting blebs. Scale bar, 5 μm. **d,e**, Live-cell confocal microscopy images of M2 blebbing melanoma cells stably expressing LifeAct–Ruby and stably transduced with shRNA targeting expression of mDia1, IQGAP1 and SPIN90 (d) and the Arp2 subunit of the Arp2/3 complex (ACTR2), NAP1 and SRA1 (e), as well as non-silencing (NS) shRNA. Scale bars, 5 μm. **f**, Distribution of bleb sizes in M2 cells stably expressing non-silencing shRNA and shRNAs targeting mDia1, ACTR2, IQGAP1, SPIN90, SRA1 and NAP1. Representative cells are displayed in (d,e). The total number of cells examined in three independent experiments (n) is indicated above each column. Comparison of non-silencing shRNA with χ²-test (Supplementary Table 4): mDia1, P = 3 × 10⁻⁴⁵; IQGAP1, P = 3 × 10⁻⁴⁵; SPIN90, P = 6 × 10⁻⁴⁵; ACTR2, P = 2 × 10⁻¹⁷; SRA1, P = 1 × 10⁻¹⁶; NAP1, P = 4 × 10⁻¹⁸ (**P < 0.01). Experiments in (b–e) were repeated three times independently with similar results. **g–i**, Percentage of HeLa cells failing to progress through the cell cycle. Statistics are derived from the total number of cells (n, indicated above each box) examined in N independent experiments. The mean for each independent experiment is shown by a black dot. Middle red bars represent medians, bounding boxes span first and third quartiles and whiskers show the range of values. Targeting siRNAs and shRNAs were compared statistically to non-silencing siRNA and shRNA with Welch’s two-sided t-test. **g**, Cells transfected with shRNA against ARPC2, SRA1 and siRNA against NAP1. ARPC2, P = 0.24; SRA1, P = 0.14; NAP1, P = 0.35. **h**, Cells transfected with siRNA against mDia1, IQGAP1 and siRNA and shRNA against SPIN90 (**P < 0.05; ***P < 0.01). mDia1, P = 9 × 10⁻⁵; IQGAP1, P = 0.026; SPIN90 siRNA, P = 1 × 10⁻¹⁵; SPIN90 shRNA, P = 0.01. **i**, Rescue experiments for SPIN90 shRNA and IQGAP1 shRNA using IQGAP1 DIB domain and full-length SPIN90 (FL-SPIN90). Statistical comparison with non-silencing shRNA: IQGAP1 shRNA + IQGAP1 DIB, P = 0.33; SPIN90 shRNA + FL-SPIN90, P = 0.7. Statistical source data can be found in Source Data Fig. 1.
Depletion of IQGAP1 did not change apparent stiffness, similar to mDia1, but surprisingly, SPIN90 depletion led to an approximately twofold increase in stiffness (Fig. 3g). Changes in apparent stiffness did not correlate with changes in cortical phosphorylated myosin light chain (pMLC) abundance (Extended Data Fig. 3 and Supplementary Table 3) or cortical density (Fig. 2e), suggesting that these changes stem from complex changes in network organization at the micro scale.

**SPIN90 is essential for cell division during embryonic morphogenesis.** Having found an unexpected role for SPIN90 in cancer

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**Fig. 2 | SPIN90 controls molecular-scale organization of cortex.** a, Representative SEM of the actin cortex at the surface of a bleb in detergent-extracted M2 cells stably expressing non-silencing shRNA or shRNA targeting NPFs. Asterisks highlight large gaps in the cortex. Scale bars, 100 nm. b, Frequency distribution of gap diameters in the cortical mesh of blebs in M2 cells. Comparison with WT using two-sided Wilcoxon’s rank test for small mesh sizes (bottom; <30 nm, **P = 0.01): Arp2, P = 0.03; Nap1, P = 0.12; mDia1, P = 0.08; IQGAP1, P = 0.04; SPIN90, P = 0.01. For large mesh sizes (top; >140 nm, **P = 0.01): Arp2, P = 0.24; Nap1, P = 0.09; mDia1, P = 0.01; IQGAP1, P = 0.01; SPIN90, P = 0.81. The full distributions are presented in Supplementary Fig. 3. c, Top: schematic representation of the measurement; cortex thickness and density are extracted from the fluorescence profiles of mCherry–CAAX (plasma membrane, red) and GFP–actin (cortex, green) in prometaphase HeLa cells. Average spatial fluorescence profiles of the plasma membrane and the actin cortex are generated (see box on right). Cortex thickness is calculated from the distance between fluorescence peaks. Bottom: representative confocal image of a prometaphase HeLa cell expressing GFP–actin (green) and mCherry–CAAX (magenta). Scale bar, 7 μm. d, Cortex thickness for prometaphase HeLa cells transfected with non-silencing siRNA or siRNA targeting IQGAP1, NAP1 or SPIN90. e, Cortex density for prometaphase HeLa cells transfected with non-silencing siRNA or siRNA targeting IQGAP1, NAP1 or SPIN90. In d, e, *P < 0.05 and **P < 0.01 compared with the appropriate control using two-sided Welch’s t-test. Cortex thickness: IQGAP1, P = 0.59; Nap1, P = 0.2; SPIN90, P = 0.02. Cortex density: IQGAP1, P = 0.03; Nap1, P = 0.26; SPIN90, P = 0.007. In b, d, e, middle red bars represent medians, bounding boxes span first and third quartiles and whiskers show the range of values. Statistics are derived from the total number of cells (n, indicated above or below each box) examined in three independent experiments. Statistical outliers are indicated by red dots. SEM data in a are representative of two independent experiments. Cortical thickness and density measurements are derived from data gathered from three independent experiments. Statistical source data can be found at Source data Fig. 2.
cells, we examined its role in embryonic tissues, where cells must frequently divide and change shape for tissue morphogenesis. Previous work has demonstrated roles for the WRC and IQGAP1 during embryonic morphogenesis as well as the presence of mDia1 in the gastrula epithelium. We therefore investigated a role for the SPIN90 orthologue in early Xenopus laevis embryos using morpholino injections (Supplementary Figs. 4 and 5).

SPIN90-depleted embryos initially developed normally, but by stage 9, they displayed multinucleated epidermal cells many times larger than epidermal cells in control embryos (Fig. 4a,b)—consistent with the role for SPIN90 in cell cycle progression observed in cancer cells (Fig. 1h,i). This phenotype eventually led to epidermis rupture and embryonic death at gastrulation. The late onset of this phenotype is perhaps due to reliance on maternal protein before mid-blastula transition or to a change in the nucleation pathway of cortical actin after ectoderm specification around stage 7 (ref. 41). Cell enlargement and multinucleation could both be rescued by co-injection of full-length mouse SPIN90 (Fig. 4c,d). Functional F-actin in SPIN90-depleted embryos at stage 9 appeared separated from the cell membrane (Fig. 4e, arrows), a phenotype that was also rescued by co-injection of full-length mouse SPIN90. Thus, SPIN90 is necessary for embryonic morphogenesis, probably via a role in cell division.

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**Fig. 3 | SPIN90 controls regrowth kinetics and mechanics.** a, Schematic of the procedure to induce single blebs by laser ablation of the cortex in metaphase HeLa cells (red arrow). Right: cells are segmented into cytoplasm, cell body cortex and bleb cortex to allow measurement of fluorescence intensity in these regions over time (Extended Data Fig. 2a–c). b, Representative actin regrowth curves in blebs induced by laser ablation in metaphase HeLa cells expressing LifeAct–Ruby transfected with non-silencing siRNA (grey lines) or SPIN90 siRNA (blue lines). The mean actin fluorescence intensity at the bleb cortex was normalized to the intensity in the cell body cortex (dashed horizontal line). Initial regrowth rates after ablation are linear with time (initial accumulation rate, dashed line). t = 0 s, ablation onset. Experiments were repeated three times independently with similar results. n = 36 cells for non-silencing siRNA and n = 16 cells for SPIN90 siRNA. c, d, Actin cortex accumulation rate in cells transfected with non-silencing siRNA or shRNA, or siRNA or shRNA targeting nucleators and NPFs. Statistical comparison to the appropriate control using two-sided Student’s t-test: ACTR2, P = 0.03; SPIN90, P = 0.001; IQGAP1, P = 2 × 10⁻⁴; SMIFH2, P = 0.06; Nap1, P = 0.001; Actin cortex accumulation rate in ns siRNA or shRNA, or siRNA or shRNA targeting nucleators and NPFs. Statistical comparison to the appropriate control using two-sided Student’s t-test: ACTR2, P = 9 × 10⁻⁵; Nap1, P = 0.002; mDia1, P = 0.001; IQGAP1, P = 2 × 10⁻⁴; SPIN90, P = 0.001. e, Principle of measurement of cortex stiffness by AFM. The pyramidal AFM tip indents the cell cortex (dark blue) to a depth of 500 nm. Controls are presented in Extended Data Fig. 2d,e. f, Cortical stiffness for control cells (DMSO, NS siRNA) and cells treated with the formin inhibitor SMIFH2 or transfected with siRNA or shRNAs targeting mDia1, IQGAP1 and SPIN90. Statistical comparison to the appropriate control using two-sided Student’s t-test: ACTR2, P = 2 × 10⁻⁶; Nap1, P = 2 × 10⁻⁵; CK666, 2 × 10⁻⁵; mDia1, P = 0.06; IQGAP1, P = 0.03; SPIN90, P = 0.0002; SMIFH2, P = 2 × 10⁻⁴; **P < 0.01. In c,d,f,g, middle red bars represent medians, bounding boxes span first and third quartiles and whiskers show the range of values. The number of independent cellular measurements (n, indicated above each box) is pooled from three independent experiments. Statistical outliers are indicated by red dots. Statistical source data can be found at Source data Fig. 3.
SPIN90 mediates crosstalk between nucleators to control network organization. Thus far, our results indicate that IQGAP1 acts as an NPF for mDia1 and that the WRC acts as an NPF for the Arp2/3 complex (Supplementary Table 3). Many of the phenotypes linked to SPIN90 depletion suggest that it acts cooperatively with mDia1 (such as bleb size, cell cycle progression, thickness and actin accumulation rate) and stiffness measurements suggest that it acts as an NPF for Arp2/3, while it presents a distinct phenotype in other

Fig. 4 | SPIN90 is necessary for correct development of Xenopus blastulae. a, Stage 9 Xenopus embryos injected with control morpholino (control-MO) or morpholino targeting SPIN90 (SPIN90-MO) along with nuclear-targeted red fluorescent protein (nRFP) and membrane-targeted GFP (mGFP). Images are representative of normal ectoderm (left) and ectoderm with mild (middle) or severe (right) defects in cell numbers and cell size. Scale bars: top row, 150 µm; bottom row, 50 µm. b, Proportion of embryos with normal ectoderm (light grey) and mild (dark grey) or severe (black) defects for different morpholino injections. Experiments were repeated four times independently with similar results. Knockdown validation is presented in Supplementary Fig. 5. c, Stage 9 Xenopus embryos co-injected with: control-MO (30 ng), SPIN90-MO (30 ng), or SPIN90-MO (30 ng) plus full-length mouse SPIN90–enhanced blue fluorescent protein (eBFP), along with nRFP and mGFP. The efficiency of the rescue was 70% with just 10% of the embryos displaying morphant phenotype, compared with ~80% morphant phenotype in SPIN90-MO treated embryos. Images are representative of three independent experiments with similar results. Scale bar, 100 µm. d, Cell area in stage 9 Xenopus embryos injected with control-MO (30 ng), SPIN90-MO (30 ng), or SPIN90-MO (30 ng) plus mouse SPIN90 mRNA. Middle red bars represent medians, bounding boxes span first and third quartiles and whiskers show the range of values. The number of independent cellular measurements (n, indicated above each box) was pooled from three independent experiments. Statistical outliers are indicated by red dots. Statistical comparison with Welch’s two-sided t-test: control-MO versus SPIN90-MO, P = 3 × 10^{-13}; SPIN90-MO versus rescue, P = 5 × 10^{-12}; control-MO versus rescue, P = 0.20. **P < 0.01. e, Stage 9 Xenopus embryos co-injected with control-MO, SPIN90-MO, or SPIN90-MO plus full-length mouse SPIN90–eBFP mRNA, along with membrane-targeted GFP. Top: phalloidin staining (white); middle: overlap with mGFP (magenta); bottom: magnified view from middle panels. In some SPIN90-MO treated cells, actin is no longer colocalized with cell membranes (white arrows). Scale bars: 100 µm (top and middle), 50 µm (bottom). Images are representative examples of three independent experiments with similar results. Statistical source data can be found at Source data Fig. 4.
Finally, when both SPIN90 and mDia1 were included, a further peak of SPIN90 and mDia1 fluorescence, as expected, since each was equally recruited when mDia1 was added to a mix of preformed filaments nucleated by spectrin–actin seeds (Fig. 6a and Extended Data Fig. 9c), and to that measured in an independent experiment with no mDia1 (Extended Data Fig. 4a) indicating that population (1) corresponds to filaments nucleated by SPIN90–Arp2/3 that did not bind mDia1. The nucleation rate of population (2) was similar to that of population (1). We thus hypothesized that these slow-then-fast growing filaments were also nucleated by SPIN90–Arp2/3 and later captured an mDia1 adsorbed on the surface. Control experiments (Extended Data Fig. 6b) indicated that such events could indeed account for a substantial fraction of population (2). The filaments in population (3) were nucleated fivefold faster than the filaments nucleated by SPIN90–Arp2/3 (Fig. 6c), consistent with the notion that they are nucleated by the ternary SPIN90–Arp2/3–mDia1 complex.

To directly observe nucleation of filaments by this ternary complex, we performed single-molecule experiments using fluorescently labelled mDia1 and SPIN90. When mixing these proteins with Arp2/3, profilin and actin (15% of which was labelled with Alexa488), we observed rapidly growing filaments bearing mDia1 at their barbed end. Even though labelled SPIN90 was less active than unlabelled SPIN90, it was present at the pointed end of these rapidly growing filaments (Fig. 7a). This observation illustrates that, as in the absence of mDia1 (Extended Data Fig. 4a), SPIN90 and Arp2/3 remain at the pointed end of filaments after nucleation (Fig. 6c–e). It was challenging to observe filament nucleation, but colocalization of SPIN90 with mDia1 could sometimes be observed prior to the appearance of fluorescently labelled actin, which then separated the peaks of SPIN90 and mDia1 fluorescence, as expected, since each protein occupies a different end of the growing filament (Fig. 7b).

Overall, our in vitro observations show that SPIN90 activates Arp2/3 to nucleate linear filaments at the expense of Arp2/3 branching, and that these filaments have an increased probability of bearing mDia1 at their barbed ends due to (at least) two key features: the efficient recruitment of mDia1 to SPIN90–Arp2/3 nucleated filaments and the formation of a ternary SPIN90–Arp2/3–mDia1 complex that greatly enhances filament nucleation. Both mechanisms result in rapidly elongating filaments with mDia1 at their barbed ends and SPIN90–Arp2/3 at their pointed ends (Fig. 7c).

**Discussion**

In this study, we identify an NPF, SPIN90, that synergizes the action of Arp2/3 and mDia1 by forming a ternary complex and has wide-ranging effects in governing actin-network architecture.
The organization of F-actin networks is central to specifying their physiological function and mechanics. Understanding what mediates the passage from one network organization to another is a key unresolved question in cell biology. Competition between nucleators can help specify structures. Indeed, the passage from the 2D-branched network topologies found in lamellipodia to the 1D topologies present in filopodia appears to be mediated by competition between actin nucleators for G-actin monomers, regulated by profilin\(^{15,46}\). We report here that SPIN90, which thus far had only been considered as a mechanism to nucleate the mother filaments for the formation of branched networks and as a means to modulate branching density (refs.\(^{15,25}\) and Fig. 5), is actually a potent enhancer of formin mDia1. SPIN90 competes with the WRC to tune the degree of Arp2/3 branching and co-opts formins to generate long

![Fig. 5](image-url)
mDia1 binds preferentially to SPIN90–Arp2/3 filaments and forms a ternary complex with SPIN90–Arp2/3 to generate fast-elongating filaments. a, In a microfluidics experiment, filaments were nucleated by surface-anchored spectrin–actin seeds or SPIN90–Arp2/3 complexes, and identified as such (Extended Data Fig. 9a,b) before flowing in a solution containing 0.5 μM G-actin (15% Alexa488-labelled), 3.5 μM profilin and 0.4 nM mDia1. Acceleration of elongation indicated the binding of mDia1. The graph shows measured mDia1-elongating filament fractions versus time and exponential fits (black lines) for filaments nucleated by spectrin–actin seeds (pink, n = 39 filaments; on rate, $k_{on} = (2.8 \pm 0.011) \times 10^{-3}$ s$^{-1}$) and by SPIN90–Arp2/3 (grey, n = 40 filaments; $k_{on} = (5.5 \pm 0.035) \times 10^{-3}$ s$^{-1}$). Two-sided log-rank test, $P = 0.01$. Shaded regions represent 95% confidence intervals. b, Three-dimensional structures of protein complexes reconstructed from electron microscopy negative stains at 27 Å resolution, from Arp2/3 incubated with mDia1 (left), SPIN90 (middle) or both (right). Electron microscopy densities were fitted using UCSF Chimera with crystal structures of Arp2/3 (PDB: 4XF2) (left) or Arp2/3–SPIN90 complex (PDB: 6DEC) (middle), or an atomic model of an mDia1 FH2 dimer (PDB: 1Y64) bound to the Arp2/3–SPIN90 complex (PDB: 6DEC) (right). Further information is presented in Extended Data Figs. 7 and 8. c, Microfluidics experiment in which a SPIN90-decorated surface is sequentially exposed to Arp2/3 then mDia1, before flowing in profilin–actin. Filaments are observed with their pointed ends attached to the surface and their barbed ends growing either slowly (bare barbed ends) or rapidly (mDia1-bearing barbed ends). d, Proportion of filaments elongating slow ((1), light blue), slow-then-fast ((2), blue) and fast ((3), dark blue) for different mDia1 concentrations, illustrated by representative kymographs. The SPIN90-decorated surface was exposed to 40 nM Arp2/3 (for 120 s), followed by 10 or 50 nM mDia1 (30 s), buffer (120 s), and then filaments were observed in the presence of 0.5 μM actin and 0.5 μM profilin. Scale bar, 5 μm. e, Nucleation of the three filament populations observed in d with 50 nM mDia1, compared to the nucleation of filaments in a chamber region unexposed to mDia1 (grey) (Extended Data Fig. 9c). Exponential fits (lines) yield nucleation rates of $8.8 \times 10^{-4}$ s$^{-1}$ for (1), $1.2 \times 10^{-4}$ s$^{-1}$ for (2) ($P = 0.1$, two-sided log-rank test) and $4.6 \times 10^{-3}$ s$^{-1}$ for (3) ($P = 2 \times 10^{-16}$, two-sided log-rank test). In d, e, n indicates the total number of filaments analysed in one experiment. Experiments were repeated twice independently in a, and three times independently in c–e, with similar results. Statistical source data can be found at Source data Fig. 6.
rapidly growing filaments to generate a rich variety of actin filament networks (Supplementary Fig. 13). Notably, such transitions were observed for ratios of SPIN90 to WRC close to those present in cells (Supplementary Table 2). Overall, our results indicate that SPIN90 regulates branching, filament growth rate and the resulting filament length, which are key parameters of the architecture of the network (Supplementary Fig. 13).

Our data reveal that SPIN90 enables synergistic action between mDia1 and Arp2/3 through at least two mechanisms, both of which result in increased nucleation of fast-growing actin filaments with SPIN90–Arp2/3 at their pointed ends and mDia1 at their barbed ends (Fig. 7c).

First, mDia1 displays an enhanced apparent on rate for the barbed ends of filaments generated by SPIN90–Arp2/3. This surprising
observation may result either from microstructural differences in the barbed ends induced at nucleation or from recruitment of mDia1 to the pointed end by binding to SPIN90–Arp2/3 followed by diffusion of mDia1 along the filament towards the barbed end, as already reported for mDia1 in a different context.

Second, we showed that SPIN90–Arp2/3 can form a ternary complex with mDia1 that increases filament nucleation fivefold compared with SPIN90–Arp2/3 alone. Our electron microscopy data (Figs. 6b) and single-filament observations (Fig. 6c–e) suggest that, following activation of Arp2/3 by SPIN90, a dimer of the FH2 domain of mDia1 binds to Arp2 and Arp3—which mimic a barbed end—to enhance nucleation and catalyse addition of monomers to the new filament. Such a ‘rocket-launching’ complex has been described for collaborative action of adenosomatous polyposis coli and mDia1, but not for Arp2/3 and mDia1, arguably the two most abundant actin nucleators. After elongation starts, our in vitro data indicate that mDia1 remains associated with the barbed end of the filament, whereas SPIN90–Arp2/3 remains at the pointed end (Figs. 6c–e and 7a,b), as already reported in the absence of mDia1. This formation mechanism takes advantage of the efficient nucleation activity of the Arp2/3 complex and rapid filament elongation by mDia1.

The total amounts of SPIN90 and other NPFs in cells can be estimated on the basis of proteomics data and measurements of cellular actin concentration (Supplementary Table 2). While determinated on the basis of proteomics data and measurements of cellular actin concentration, the concentrations of available, active proteins in the cortical range (Supplementary Table 2).

Overall, our results show that the activity of the two main cortical actin nucleators, mDia1 and Arp2/3, is modulated by the interplay of three NPFs to finely tune cortical actin organization, kinetics and mechanics.

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Methods

Cell culture and generation of cell lines. M2 melanoma cells were a gift from T. Stossel (Harvard Medical School) and were described in ref. 1. Cell lines were cultured in MEM with Earle’s salts and l-glutamine (Gibco) with penicillin–streptomycin, and 10% fetal bovine serum (FBS). F20 were obtained from the Mammalian Gene collection or SPIN90 fetal bovine serum, l-glutamine and penicillin–streptomycin. 10% fetal calf serum or in phenol red-free DMEM (Gibco) supplemented with 10% Meers. Weiner (University of California San Francisco). eBFP253 was obtained from University). WAVER–GFP with a truncated CMV promoter was a gift from O. Bowman (Göttingen, Germany), and Fli-I–GFP52 was a gift from R. Tombes (Virginia Commonwealth University). The stable HeLa GFP–actin line and the HeLa LifeAct–Ruby line were a gift from the MPI–CBG Technology Development Studio. The stable HeLa GFP–actin line and the HeLa LifeAct–Ruby line were described in ref. 1. These cells were cultured in DMEM (Gibco) with penicillin–streptomycin, l-glutamine, 10% fetal bovine serum and 750 μg/ml G418. Cells were arrested in prometaphase with 100 nM nocodazole (Merck Biosciences) for 16 h for the localization studies. For metaphase arrest in laser-ablation and AFM studies, cells were treated for at least 1 h with 10 μM MG132 (Sigma).

To obtain stable protein knockdown, M2 or HeLa cells (wild type or LifeAct–Ruby) were transfected with lentiviruses expressing shRNAs constructs targeting the genes of interest (see Supplementary Information) or transfected with linearized cDNA (see Supplementary Information). The cells were then selected with 250 ng/ml puromycin for 2 weeks. Flow cytometry was performed to obtain homogenous levels of expression (see Supplementary Information).

None of the cell lines in this study were found in the database of commonly misidentified cell lines maintained by International Cell Line Authentication Committee and National Center for Biotechnology Information BioSample. All cell lines were cultured at 37 °C with 5% CO2. All lines were routinely screened for the presence of mycoplasma using the mycoALERGT kit (Lonza). Where not otherwise stated, cells were cultured in L-15 medium (Gibco) supplemented with 10% fetal calf serum in phenol red-free DMEM (Gibco) supplemented with 10% fetal bovine serum, l-glutamine and penicillin–streptomycin.

Plasmid construction and transfection. Full-length human and mouse SPIN90 (also known as NCAPSD) were obtained from the Mammalian Gene collection or the LM-CEGE library and cloned into EFGP-C1, EGF-N1 or EBFP2-C1 vectors using restrictions sites inserted by PCR. SPIN90, SPIN90-NT, and SPIN90-CT were described in ref. 6. IQGAP1–GFP was a gift from D. Sacks (National Institutes of Health), the DBR domain of IQGAP1 tagged with GFP6 was a gift from R. Grosse (University of Marburg, Germany), and Fib1–GFP was a gift from R. Tombs (Virginia Commonwealth University). WAVE2–GFP with a truncated CMV promoter was a gift from O. Weiner (University of California San Francisco). EBFP2 was obtained from Addgene (plasmid 14893) and substituted for eGFP in some constructs. All gene products were verified by sequencing.

cDNA was purified from bacteria using the Qiagen Spin Miniprep Kit. Transfections were carried out using Lipofectamine 2000 according to the manufacturer’s instructions.

Confocal microscopy. All fluorescence imaging (except for cortex-ablation experiments) were performed using a ×100 oil-immersion objective on an inverted microscope (IX-81, Olympus) fitted with a spinning-disk head (Yokogawa, CSU2). Images were acquired with an Andor iXon camera and analysed using ImageJ (http://rsweb.nih.gov/ij/).

Excitation with a 488 nm laser was utilized for GFP-tagged proteins, with a 543 nm laser for mRFP, Ruby- and mCherry-tagged proteins as well as Alexa555, labelled anti-α-tubulin, with a 405 nm laser for EBFP2 tagged proteins, and with a 647 nm laser labelled with CellTracker Deep Red (Life Technologies).

SEM of the cortex of blebs. Sample preparation for SEM was performed as described7 with minor modifications as described. Two hours before sample preparation, whole cells were plated onto 12 mm glass coverslips. Immediately before fixation, the coverslips were washed three times with L-15 without serum and transferred to cytoskeleton buffer (50 mM imidazole, 50 mM KCl, 0.5 mM MgCl2, 0.1 mM EDTA, 1 mM EGTA, pH 6.8) containing 0.5% Triton-X and 0.25% glutaraldehyde for 5 min. This was followed by a second extraction with 2% Triton-X and 1% CHAPS in cytoskeleton buffer for 5 min before washing the coverslips in cytoskeleton buffer three times. The remainder of the protocol was identical to the originally described procedure8. The cells were dehydrated with serial ethanol dilutions, dried in a critical point dryer, coated with 5–6 nm platinum–palladium and imaged using the in-lens detector of a JEOL7401 Field Emission Scanning Electron Microscope (JEOL). All samples were prepared in duplicate and images from two independent experiments were acquired. Similar phenotypes were observed in each independent experiment and images of at least eight different cells were acquired for each experimental condition.

Cortical thickness measurement. Measurement of the thickness of the actin cortex in metaphase HeLa cells was as described9. In brief, two–colour–imaging stacks (30–40 μm) acquired at 2 μm intervals were acquired around the equatorial plane of rounded cells using a ×60 colour–corrected objective (1.40 numerical aperture, OSC2 PlanApo) mounted on an Olympus FV1200 microscope. After correcting for chromatic shift and magnification using custom software written in MATLAB, a single equatorial plane was selected for each image using Fiji image analysis software. Cortex thickness and density were extracted as described10. For membrane width measurements, we measured the full width at half-maximum of the plasma membrane intensity peak by interpolating the x position on the line scan on either side of the peak by linear interpolation of the two closest points. The half-maximum was defined as half of the difference between the peak intensity and intracellular background.

Actin regrowth speed analysis. The rate of actin accumulation during cortex retraction was measured as described11. Blebs were induced by exposure of a small region of the cortex of metaphase HeLa cells expressing LifeAct–Ruby to UV pulses. Following induction, blebs grow rapidly before stopping and eventually retracting. Actin is initially absent from below the bleb membrane but progressively accumulates as growth slows and retraction starts. To allow for reliable segmentation of the cell contour for subsequent image analysis, we added 2 μM latrunculin A to the extracellular medium. Latrunculin A is a potent, highly specific, actin filament severing agent that was used to intensely label the actin cytoskeleton with a monolayer of fluorescent actin filaments. This allowed segmentation of the cortex from the cytoplasm and segmentation of the induced bleb from the rest of the cell body. Cortical and cytoplasmic fluorescence intensities of actin could then be monitored in the bleb and compared with the cell body control value. This analysis yielded values relating the evolution of the actin fluorescence intensity in the bleb normalized to the cortical fluorescence intensity in the cell body. Actin accumulation in the cortex displayed two markedly different phases: the first started immediately after laser ablation and ended shortly after the cessation of growth; the second started after growth had finished and ended after bleb retraction (Extended Data Fig. 2b). Actin accumulation is approximately linear in both regimes and slopes relating the percentage actin accumulation per second could be measured by fitting straight lines to each interval.

AFM and data analysis. Indentations of cells by AFM were performed using a JPK NanoWizard-1 AFM (JPK) mounted on an inverted microscope (IX-81, Olympus). The day before the experiment, cells were plated onto 35 mm glass-bottom Petri dishes. Cells were incubated in MG132 (10 μM, Sigma) for 2 h before measurement to arrest cells in metaphase. Experiments were performed at room temperature and cells were maintained in Leibovitz L-15 medium (Life Technologies) supplemented with 10% FBS (Sigma-Aldrich) and MG132 (10 μM). Before each experiment, the spring constant of the cantilever was calibrated using the thermal noise implemented in the AFM software (JPK SPM). The sensitivity of the cantilever was measured from the slope of force–distance curves acquired on glass. For apparent stiffness measurements, we used soft cantilevers with V-shaped tips (BioLeVer OBL-10, Bruker; nominal spring constant of 0.006 N m−1).

For each measurement, the cantilever tip was first aligned above a metaphase cell using the optical microscope. Then, force–distance curves were acquired over the centre of the cell at the 4 vertices of a square with a 2 μm side. At each of these four positions, up to 10 curves were acquired with an approach speed of 2.5 μm s−1 and a target force of 2.5 nN. Force–distance curves were then post-processed to compute an apparent stiffness. First, we determined the contact point between the cantilever tip and the cell using the method described9, implemented in MATLAB (MathWorks). The indentation depth was then calculated by subtracting the cantilever deflection d from the piezo displacement beyond the contact point z (d = z – d). The resultant force–indentation curves were then averaged over each position and fitted with the Sneddon model to calculate each location’s apparent elasticity. Curves fitted with the Sneddon model were restricted to indentation depths lower than 500 nm to maximize contributions of the cortex to the restoring force and minimize contributions from the cytoplasm.

Electron microscopy and single–particle analysis. Concentrated mixtures of either Arp2/3–mDia1, Arp2/3–SPIN90, or Arp2/3–SPIN90–mDia1 were diluted and applied to freshly glow–discharged, 300 mesh carbon–coated copper grids at final concentrations 22 nM Arp2/3, 100 nM SPIN90 and 50 nM mDia1. The samples were negatively stained with uranyl formate 1%. Data were collected with a FEI tecnai G2 transmission electron microscope equipped with a LaB6 emission filament operating at 200 kV. Images were captured on a TVIPS F416 CMOS camera at x30,000 magnification and 1.5–2.5 μm underfocus. The pixel size used was 3 Å per pixel. Using a Leica TRISTAR transfer function estimations from CTFIND5,26,27, micrographs were phased.

Particle hands were picked using XMIPP software from the image processing framework SCIPION10. Particles whose size would correspond to that of a potential complex including Arp2/3 were selected. A total of 7,572 particles were selected for the mixture of Arp2/3–mDia1, 5,618 for the mixture of Arp2/3–SPIN90 and 10,044 particles for the mixture of Arp2/3–SPIN90–mDia1. Two–dimensional class averages were obtained in Relion 3.01. Particle belongings to blurred averages were excluded from further analysis.

3D classification was performed to sort three classes using Relion 3.0. Low–pass–filtered crystal structures were used as references (low–pass filters 40 Å). The conformation of Arp2/3–SPIN90 complex11 (PDB: 6DEC) was used for Arp2/3–SPIN90 data analysis. Different models generated from the Arp2/3–SPIN90 complex (PDB: 6DEC) and FH2 domains from Bni1–actin complex12 (PDB: 1Y64) were used for Arp2/3–SPIN90–mDia1 data analysis. Arp2/3 crystal structure12 (PDB: 4XF2), with and without FH2 domains from
Bni1p–actin complex (PDB: 1Y64), were used for Arp2/3–mDia1 data analysis. The particles belonging to the 3D class displaying the best fit to the model complex used as reference were selected for further processing, while the divergent ones were left out.

These sorted particles were then used to reconstruct a 3D model with Rebuild 3.0 using strongly low-pass filtered references to prevent any bias (low-pass filter: 50–60 Å). The resulting 3D reconstructions shown in Fig. 6b and Extended Data Fig. 7 have a resolution of 27 Å for Arp2/3–mDia1 (5,456 particles used), Arp2/3–SPIN90 (4,690 particles used) and Arp2/3–SPIN90–mDia1 (2,006 particles used). To interpret the resulting 3D reconstructions, the non-filtered crystal structures used as references were fitted in the corresponding 3D maps using UCSF Chimera. Spurious noise from electron microscopy densities was hidden within the ‘hide dust’ command in UCSF Chimera to facilitate readability.

Image acquisition for in vitro experiments. The microfluidic devices or open flow chambers were placed on a Nikon TiE inverted microscope, equipped with a x60 oil-immersion objective. An objective heater (Okolab) maintained the temperature at 25 °C on the coverslip. The total internal reflection microscopy (TIRFM) setup was controlled with Metamorph, illuminated by 100 mW tunable lasers (ILAS2, Roper Scientific, now Gataca Systems). Images were acquired on an Evolve EMCCD camera (Photometrics). Image software was used to analyse images.

For single-filament assays (Fig. 5a–c, Extended Data Figs. 4a,b, 6b, 9 and Supplementary Fig. 6f), microfluidics experiments were carried out with polystyrene and polydimethylsiloxane (PDMS) (height 18, 200 µm) devices with dimensions of 60 × 800 × 1 mm, based on the original protocols4,5. The devices are cross-shaped, consisting of channels with three inlets and one outlet. Flows were controlled and monitored by an MFCS and flow units (Fluigent).

Polydimethylsiloxane chambers were cleaned and mounted by following the protocol described7. Single-filament experiments were performed in F-buffer (5 mM Tris-HCl pH 7.8, 1 mM MgCl2, 0.2 mM EGTA, 0.2 mM ATP, 10 mM DTT, 0.5 mM 1,5-diaminobenzene, 50 mM KCl and 0.1% BSA). The surface was passivated with 3% BSA, 0.7 µM 15% Alexa488–actin, and 0.5 µM 15% Alexa647–actin was flowed into the chamber to generate actin filaments. Then, the surface was briefly exposed to 100 µM iodoacetate, which may sever filaments. This yielded a surface with filaments with a broad length distribution anchored to it. These filaments were then exposed to 0.4 nM mDia1, 0.5 µM 15% Alexa488–actin and 3.5 µM profilin simultaneously to compare affinity of mDia1 for long and short filaments.

To measure mDia1 binding to actin filaments of different lengths (Extended Data Fig. 9b), spectrin–actin seeds were attached to the coverslip surface. After 90 s, the surface was passivated with 3% BSA, 0.7 µM 15% Alexa488–actin, and 0.5 µM 15% Alexa647–actin was flowed into the chamber to generate actin filaments. Then, the surface was briefly exposed to 100 µM iodoacetate, which may sever filaments. This yielded a surface with filaments with a broad length distribution anchored to it. These filaments were then exposed to 0.4 nM mDia1, 0.5 µM 15% Alexa488–actin and 3.5 µM profilin simultaneously to compare affinity of mDia1 for long and short filaments.

To interpret the resulting 3D reconstructions, the non-filtered crystal structures (Fig. 7) have a resolution of 27 Å for Arp2/3–mDia1 (5,456 particles used), Arp2/3–SPIN90 (full length or C-terminus) and without mDia1, a GST–SPIN90–decorated surface in a microfluidic chamber was exposed to 40 nM Arp2/3 complex for 2 min and rinsed, followed by exposure to 10 nM (or 50 nM) mDia1 for 30 s. The surface was then rinsed with buffer for 2 min before being exposed to profilin–actin (0.5 µM 15% labelled Alexa488–actin and 0.5 µM profilin). The nucleation and the elongation rates of the observed filaments were monitored (Extended Data Fig. 9c).

To compare the nucleation rates of SPIN90–Arp2/3 complex in regions with and without mDia1, a GST–SPIN90–decorated surface in a microfluidic chamber was exposed to 40 nM Arp2/3 complex for 2 min and rinsed; we then exposed only half of the chamber to 50 nM mDia1 by manipulating the input pressures in our microfluidic device. The surface was then rinsed with buffer for 2 min before being exposed to 50 nM mDia1 and 0.5 µM 15% Alexa488–actin and 0.5 µM profilin (Extended Data Fig. 9d).

For measuring mDia1 binding to actin filaments of different lengths (Extended Data Fig. 9c), to specifically anchor GST–SPIN90–Arp2/3 and spectrin–actin seeds were attached to the coverslip surface. After 90 s, the surface was passivated with 3% BSA, 0.7 µM 15% Alexa488–actin, and 0.5 µM 15% Alexa647–actin was flowed into the chamber to generate actin filaments. Then, the surface was briefly exposed to 100 µM iodoacetate, which may sever filaments. This yielded a surface with filaments with a broad length distribution anchored to it. These filaments were then exposed to 0.4 nM mDia1, 0.5 µM 15% Alexa488–actin and 3.5 µM profilin simultaneously to compare affinity of mDia1 for long and short filaments.

To investigate direct binding between SPIN90 and mDia1 (Extended Data Figs. 4c and 6b), we exposed the GST–SPIN90-decorated surface sequentially to 40 nM Arp2/3 for 2 min and rinsed, exposed to 0.4 nM mDia1 for 2 min, then exposed to 0.5 µM Alexa488–actin, and 0.5 µM profilin simultaneously to compare affinity of mDia1 for long and short filaments.
were washed by adding 500 μL GST pull-down buffer. After centrifugation at 500g for 1 min, the supernatant was discarded. After the washing steps were repeated three times, proteins attached to beads were eluted with 30 μL 20 mM reduced glutathione. The sample was separated by SDS–PAGE for analysis by western blot (Extended Data Fig. 4c and Supplementary Fig. 12). Pulled-down SPIN90 was identified by anti-His antibody (QIAGEN).

To investigate formation of a ternary complex between SPIN90, the Arp2/3 complex and mDia1, we carried out a pull-down assay with SPIN90 anchored to glutathione beads. Fifty microlitres of 12 μM Glutathione Sepharose 4B decorated with full-length GST-SPIN90 was mixed with 0–540 nM Arp2/3 for 1 h at 4 °C. After washing 3 times with 300 μL GST pull-down buffer, these beads were further incubated with 700 nM His-tagged mDia1 dimer for 1 h at 4 °C. After washing the beads 3 times with 300 μL GST pull-down buffer, proteins attached to beads were eluted with 30 μL 20 mM GSH. The sample was separated by SDS–PAGE for western blot analysis. His (QIAGEN) and ArpC2 (Sigma) antibodies were used to detect His-tagged mDia1 and Arp2/3, respectively. Membranes were imaged with ImageQuant LAS-4000 Mini Imaging System. Pulled-down mDia1 was quantified using ImageJ (Extended Data Fig. 6a and Supplementary Fig. 12).

The control experiment (Supplementary Figs. 8c and 12) was done in the same way except in the first step, 50 μL of 12 μM Glutathione Sepharose 4B decorated with GST or GST-SPIN90 C-terminal construct was mixed with or without 540 nM Arp2/3 for 1 h at 4 °C.

Statistics and reproducibility. Phenotype distribution after gene depletion was compared with cells stably expressing non-silencing shRNA using a χ²-test (Supplementary Table 3). Cells were imaged from at least two independent experiments; P < 0.01 was deemed statistically significant. The amount of cell death was compared using Welch’s two-sided t-test with unequal s.d.; P < 0.05 was deemed statistically significant. Changes in the proportion of gaps of a given size across conditions was compared with a two-sided Wilcoxon’s rank test; P < 0.01 was deemed statistically significant. Changes in cortical thickness, cortical density and cell area were examined using two-sided Welch’s t-test; P < 0.05 was deemed statistically significant. Changes in actin accumulation rates and cortical stiffness across conditions were examined using two-sided Student’s t-tests; P < 0.01 was deemed statistically significant. Changes in cortical pH were compared across conditions using one-way ANOVA on ranks. P < 0.01 was deemed significant. Changes in actin nucleation rates of different filament populations were compared with a two-sided log-rank test; P < 0.05 was deemed significant. Changes in mDia1 binding to filaments that nucleated differently or with different lengths, measured with the microfluidic device, were compared with two-sided log-rank test; P = 0.05 was deemed significant. Changes in mDia1 binding to mother and daughter filaments, or to filaments nucleated differently, measured with an open chamber, were compared with Pearson’s χ² test; if one of the populations was too small (<5 individual samples), then the changes were compared with a one-sided Fisher’s exact test; P < 0.05 was deemed significant. Changes in actin nucleation rates of different filament populations were compared with a two-sided log-rank test. P < 0.05 was deemed significant. All experiments were repeated at least twice independently with similar results. The exact number of repeats for each experiment is indicated in the relevant figure legend.

Reporting Summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability
Mass spectrometry data have been deposited in ProteomeXchange with the primary accession code PXD018318. All data supporting the conclusions of this paper are available from the authors upon reasonable request.

Code availability
The code used to analyse data in this study is available from the authors upon request.

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Author contributions
G.C., G.R.-L., A.J., A.Y., L.C. and M.V. designed the experiments. A.Y. carried out the laser ablation, AFM and fluorescence-quantification experiments. M.V. carried out all scanning electron microscopy on cells, long-term microscopy and bleb-size characterization. M.V. carried out all laser ablation, AFM and fluorescence-quantification experiments. L.C., G.R.-L. and A.J. carried out in vitro experiments. P.C. did all measurements of cortex thickness and F-actin density. M.B.S. designed the software for analysis of SEM images. G.L., A.M. and P.P.R. performed all proteomic and quantitative PCR experiments and analysed data. A.Y. and E.F. carried out all western blotting. G.C. generated plasmid constructs and cell lines and carried out immunostaining. J.M. and A.B. carried out the molecular-scale electron microscopy and generated the complex structures. M.B. contributed live-imaging constructs and some imaging experiments. A.A. provided plasmid constructs. E.H.B. and R.M. contributed Xenopus experiments. E.K.P provided conceptual advice. G.C. and G.R.L. wrote the manuscript. All authors discussed the results and the manuscript.

Competing interests
The authors declare no competing interests.

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Extended Data Fig. 1 | Localisation of NPFs in blebbing melanoma cells. (a–d) Confocal microscopy images of blebbing M2 melanoma cells expressing GFP-tagged NPFs. White arrowheads indicate expanding blebs and red arrowheads retracting ones. Scale bars = 5 μm. Experiments were repeated two times independently with similar results. a, IQGAP1 localizes to the rim of retracting blebs (red arrowhead) but not to the rim of nascent blebs (white arrowhead). b, SPIN90-GFP before (left) and after (right) permeabilization and fixation to remove cytoplasmic signal. Following permeabilization and fixation, SPIN90 remains localized to the actin cortex of retracting blebs but is absent from the cortex of nascent blebs. c, FLII-GFP before (left) and after (right) permeabilization and fixation. Following permeabilization and fixation, FLII remains localized to the actin cortex of retracting blebs. d, Constitutively active cortactin-GFP localizes to the rim of retracting blebs but not to the rim of nascent blebs.
Extended Data Fig. 2 | See next page for caption.
Extended Data Fig. 2 | Control experiments for actin accumulation rate and cortical stiffness. **a**, Representative laser ablation experiment. All images are a single confocal section. Top row: Alexa647 is added to the medium for robust segmentation of the cell. Bottom row: LifeAct Ruby. Left column: cell before ablation, middle column: after ablation, right column: segmented image. Timings are indicated on the top row images. Scale bar=10 μm. **b**, Representative actin regrowth curve in a bleb induced by laser ablation as in a. Pink curve: evolution of mean cortical actin fluorescence in the bleb normalized to the mean cortical intensity in the cell body (pink). Blue curve: evolution of bleb area normalized to cell body area. Initial regrowth rates are linear with time (initial accumulation rate). t = 0 s, ablation onset. *(c-e)* Data plotted as box-whisker plots. The distributions' medians, first and third quartiles, and ranges are represented by the central red bars, bounding boxes and whiskers, respectively. Statistics are derived from the total number (n, indicated above each box) of cells examined in three independent experiments. Each dot represents one cell measurement. Statistical outliers are indicated by red dots. "p < 0.01 compared to the appropriate control. **c**, Actin accumulation rate in metaphase cells at 37 °C and Room Temperature. Two-sided Student t-test: 37 °C vs RT: p = 0.19. **d**, Apparent elastic modulus for cells expressing Non-Silencing shRNA (NS sh), transfected with Non-Silencing siRNA (NS si), and treated with DMSO. One-way ANOVA on ranks: NS siRNA vs NS shRNA: p = 0.001, DMSO vs NS shRNA: p = 0.001. DMSO vs NS siRNA: p = 0.99. **e**, Apparent elastic modulus of WT and ACTR2 shRNA cells treated with DMSO and blebbistatin (Bb). One-way ANOVA on ranks: WT DMSO vs WT blebbistatin: p = 2.10^-9; WT DMSO vs ACTR2 shRNA DMSO: p = 4.10^-5; ACTR2 shRNA DMSO vs ACTR2 shRNA blebbistatin: p = 5.10^-13; WT blebbistatin vs ACTR2 shRNA blebbistatin: p = 0.71. Statistical source data can be found at Source data figure ED2.
**Extended Data Fig. 3 | Regulation of myosin localization and phosphorylation in cells with nucleator or NPF depletion.**

**a.** Representative pMLC distribution in metaphase HeLa cells visualized by immuno-staining for different protein depletions and for non-silencing (NS) siRNA. Each image is a single section of a confocal microscopy stack and is shown in inverted contrast. **b.** Mean cortical pMLC fluorescence intensity for different treatments normalized to the mean cortical pMLC fluorescence intensity for non-silencing (NS) siRNA or shRNA. The distributions’ medians, first and third quartiles and ranges are represented by the central red bars, bounding boxes and whiskers, respectively. Statistics are derived from the total number (n, indicated above each box) of cells examined in three independent experiments. Each dot represents one cell measurement. Statistical outliers are indicated by red dots. Statistical comparisons of the means were performed using one-way ANOVA on ranks compared to NS siRNA/shRNA. ACTR2 siRNA: p = 7 × 10^{-5}, NAP1: p = 0.03, mDia1: p = 0.85, IQGAP1: p = 0.08, SPIN90: p = 0.74. **p < 0.01 compared to the appropriate control. See Supplementary Figure 9 for controls.**

**c.** Change in cortical myosin regulatory light chain fluorescence intensity upon treatment with DMSO (left panel) or CK666 (right panel). In each panel, the top row shows the fluorescence intensity before treatment and the bottom row after treatment for the same cell. The left most column shows myosin regulatory light chain fluorescence (MRLC-GFP), the middle column shows LifeAct-Ruby, and the right column shows the overlay with MRLC in green and LifeAct in Magenta. Experiments were repeated twice independently with similar results. (a,c) Scale bars=10 μm. Statistical source data can be found at Source data figure ED3.
**Extended Data Fig. 4 | Interaction of SPIN90 with single nucleators.**

**a.** Sketch of a microfluidics experiment, where surface-anchored SPIN90 is exposed to 30 nM Arp2/3 complex for 5 min, followed by profilin-actin (15% Alexa488 labeled). TIRF microscope image: filaments nucleate and grow from their free barbed ends while their pointed ends remain attached. Plot: appearance of 217 filaments over time (red dots), with an estimated nucleation rate of $9.5 \times 10^{-4}$ s$^{-1}$ (exponential fit, solid line).

**b.** Microfluidics experiment where surface-anchored SPIN90 is exposed to 50 nM mDia1 for 5 min, followed by profilin-actin (15% Alexa488 labeled) and then profilin-actin (unlabeled). Kymograph (from TIRF microscopy): filaments grow rapidly from their anchored barbed ends. Plot: elongation rate of these filaments (blue, $n=17$ measured filaments) compared to filaments growing from mDia1 directly anchored to the surface without SPIN90 (red, $n=17$ measured filaments). Boxes indicate averages and standard deviation, for each group of $n$ filaments.

**c.** GST pull down assay to detect binding between mDia1 and SPIN90. GST or GST-fused mDia1 coated beads were incubated with various concentrations of His tag-fused SPIN90 for 1 hour. Proteins attached to the beads were eluted with 20 mM GSH. The presence of SPIN90 was detected by western blot. The experiments were repeated three times independently with similar results. Uncropped Western blot can be found in Source data figure ED4.

**d.** Density of rapidly growing filament barbed ends (i.e. bearing a formin), observed over 74752 $\mu$m$^2$ after 300 s in the experiment shown in Fig. 5C. The error bars show the 95% confidence interval. Statistical source data can be found at Source data figure ED4.
Extended Data Fig. 5 | mDia1 preferentially binds to filaments nucleated by SPIN90-Arp2/3. a, Comparison of mDia1 binding to mother filaments versus branches. Preformed branched actin filaments (15% Alexa568 labeled) were mixed with 0.5 μM G-actin (15% Alexa488-labeled), 0.5 μM profilin, 0.4 nM mDia1, before being introduced into a passivated open chamber. The TIRF microscopy image shows Alexa568 in red and Alexa488 in green. Plot: fraction of rapidly growing barbed ends (i.e. bearing a formin) observed after 90 s, within the population of mother filaments (dark blue bar) and branches (light blue bar). Comparison with Pearson’s chi square test: \( p = 0.88 \). b, Comparison of mDia1 binding to spontaneously nucleated versus SPIN90-Arp2/3-nucleated filaments. Spontaneously nucleated actin filaments (15% Alexa568-labeled) were mixed with SPIN90-Arp2/3-nucleated actin filaments (15% Alexa488-labeled) and with 0.2 nM mDia1, 0.5 μM profilin and 0.5 μM G-actin (3% Alexa488-labeled). TIRF microscopy image: filaments growing from spontaneously assembled seeds (red arrow) or from SPIN90-Arp2/3-nucleated seeds (green arrow) are identified by the fluorescence at their pointed end. Plot: fraction of rapidly growing barbed ends observed after 300 s, within each population. Comparison with a Pearson’s chi square test: \( p = 0.009 \). Similar results were observed when repeating the experiment and inverting the fluorophores (Supplementary Figure 8B). c, Comparison of the fraction of mDia1-bearing barbed ends depending on filament nucleation history. SPIN90-Arp2/3-nucleated actin filaments (15% Alexa568 labeled) were mixed with 0.5 μM G-actin (15% Alexa488 labeled), 0.5 μM profilin, 25 nM Arp2/3, 250 nM SPIN90 and 0.2 nM mDia1. TIRF microscopy image: filaments nucleated before mixing (i.e. exposed to mDia1 after their nucleation by SPIN90-Arp2/3) are identified thanks to their Alexa568 (red) pointed end region. Plot: fraction of rapidly growing barbed ends observed after 300 s, within each population. Comparison with a Pearson’s chi square test: \( p = 9 \times 10^{-7} \). (a, b, c) Error bars represent 95% confidence intervals. **\( p < 0.01 \) and ***\( p < 0.001 \), Pearson’s chi-squared test. For each population, \( n \) indicates the number of analysed barbed ends, in one experiment. The experiments (A, B) were repeated twice, and (C) three times, independently with similar results. Statistical source data can be found at Source data figure EDS.
Extended Data Fig. 6 | mDia1 binds to the SPIN90-Arp2/3 complex. a, mDia1 is dose-dependently eluted with Arp2/3 bound to GST-SPIN90 decorated beads. Left: Anti-His and Anti-ArpC2 western blots of GST pull down assay. GST beads were incubated with 12 μM GST-SPIN90 and with the indicated amounts of Arp2/3. The beads were washed and incubated with 750 nM mDia1. Right: quantification of pulled-down mDia1, normalized to GST-SPIN90 without Arp2/3 (mean ± SD, n = 3 independent experiments). Black dots show the data points. For each concentration, the red dot shows the mean and the red bars the standard deviation. Uncropped Western blot can be found in Source data figure ED6. b, Left: schematic diagram of a control experiment (related to Fig. 6C-D). Control experiments consist in observing the elongation of new, bare barbed ends growing in a microfluidics chamber with a SPIN90-decorated surface exposed to Arp2/3 and mDia1. This is achieved by first following the sequence shown in Fig. 6C, and then photo-severing the filaments with a strong laser illumination (see Methods). We then observed the regrowth of filaments from SPIN90-Arp2/3. The photo-severing ensured that the newly elongating barbed ends were initially without mDia1. The distribution of slow (light blue), slow then fast (blue), and fast (dark blue) filaments was compared to experiments like the ones presented in Fig. 6C-D (bar charts, on the right, n = number of filaments). “Complex” denotes the experiments performed as in Fig. 6C-D (with an additional exposure to light, to have the same conditions as in the control, see Methods) and “control” denotes the control experiments. Comparison using one-tailed Fisher’s exact test: p = 0.31 for the slow-then-fast population, and p = 0.0013 for the fast population. n is the number of filaments randomly picked and investigated in an independent experiment. The experiment was repeated twice independently with similar results. Statistical source data can be found at Source data figure ED6.
Extended Data Fig. 7 | Visualisation of different orientations of the protein complexes formed by Arp2/3, SPIN90, and mDia1. Different combinations of complexes (binary or ternary) involving the Arp2/3 complex, SPIN90, and mDia1 were analyzed by electron microscopy and single particle analysis. For each protein complex, three-dimensional reconstructions were obtained from 3D classifications and compared to existing or generated crystal structures (see methods). a, Incubation of mDia1 and Arp2/3 resulted in a 3D structure which only accommodates Arp2/3, suggesting that mDia1 and Arp2/3 do not interact when SPIN90 is absent. b, Conversely, mixing the Arp2/3 complex and SPIN90 results in a complex for 76% of the particles. Within the complex, SPIN90 (green) clearly appears as an additional density when compared with the truncated docked crystal structure. c, When compared with the Arp2/3-SPIN90 complex in B, the 3D envelope (resolution of 27 Å) resulting from the ternary complex (SPIN90-Arp2/3-mDia1) exhibits an additional density accommodating a dimer of FH2 domains for 22% of the particles. (a-c) Each protein complex is subjected to a variety of rotations to visualize its full structure.
Extended Data Fig. 8 | Raw data of negatively stained Arp2/3, SPIN90, mDia1 mixes. Scale bars: 100 nm in upper images, 15 nm in middle images; lower boxes are 29.8 nm².  

**a**, Micrograph of Arp2/3 mixed with mDia1 (top), subselection of particles used for Arp2/3 3D reconstruction (middle), and examples of 2D classes (bottom).  

**b**, Micrograph of Arp2/3 mixed with SPIN90 (top), subselection of particles used for Arp2/3-SPIN90 3D reconstruction (middle), and examples of 2D classes (bottom).  

**c**, Micrograph of Arp2/3 with SPIN90 and mDia1 (top), subselection of particles used for Arp2/3-SPIN90-mDia1 3D reconstruction (middle), and examples of 2D classes (bottom).
Extended Data Fig. 9 | Microfluidic experiments examining interactions of mDia1 with preformed filaments and with SPIN90-Arp2/3. **a**, Sketch of microfluidics experiment related to Fig. 6A, showing how two groups of filaments generated by different nucleators were generated and identified. SPIN90 and spectrin-actin seeds were anchored to the surface (1st row), and exposed to 0.7 µM 15% Alexa488 G-actin to generate filaments nucleated from the seeds (2nd row). Then Arp2/3 was flowed in (3rd row), followed by 0.7 µM 15% Alexa488 G-actin to generate SPIN90-Arp2/3-nucleated filaments (4th row). Since all the spectrin-actin seeds had already nucleated filaments during the first exposure to actin, the newly nucleated filaments were identified as nucleated by SPIN90-Arp2/3. Finally, mDia1 was introduced into the chamber with profilin-actin (5th row).

**b**, mDia1’s binding to filaments nucleated by spectrin-actin seeds with different lengths, related to Fig. 6A. To check whether mDia1 bound differently to filaments with different lengths, related to Fig. 6A. To check whether mDia1 bound differently to filaments with different lengths, filaments nucleated by spectrin-actin seeds in a microfluidics setup, with heterogeneous lengths, were exposed to 0.4 nM mDia1, 0.5 µM 15% Alexa488 G-actin and 3.5 µM profilin. The binding of mDia1 to long filaments (>5 µm, n = 40 filaments) was compared to that for short filaments (<1 µm, n = 39 filaments), by plotting the percentage of fast-growing filaments in each population over time. The experiment was repeated twice independently, with similar results. Our results indicate no impact of filament length on mDia1 binding (p = 0.6, two sided log-rank test). Shaded regions, 95% confidence intervals.

**c**, Sketch detailing the observation of the mDia1-free population reported in Fig. 6E. After the SPIN90-decorated surface was exposed to Arp2/3, we exposed only one half of the chamber to mDia1 (1). After rinsing with buffer (2), the surface was exposed to profilin-actin. In the region exposed to mDia1, filaments with different elongation rates appeared; while in the region that was not exposed to mDia1, only slow growing filaments were observed. Statistical source data can be found at Source data figure ED9.
Reporting Summary

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Software and code

Policy information about availability of computer code

Data collection
- Confocal microscopy images were acquired using Olympus FV 10 ASW software v3.0 or the Andor IQ software version 1.0 and 3.0.
- AFM data were acquired using JPK SPM Desktop v5.0.1250.
- Long term imaging data was acquired using Zeiss ZEN software v3.0.
- Scanning electron microscopy data was acquired using the JeolJCM acquisition software v1.
- Mass Spectrometry data was acquired using a high resolution hybrid mass spectrometer LTQ orbitrap XL and Scaffold v4.8.9.
- Western blots were acquired on a ChemiDoc XRS+ System (Bio-rad) or an ImageQuant LAS 4000 system.
- The TIRF setup was controlled by Metamorph v7.8. Images were acquired on an Evolve EMCCD camera (Photometrics).
- Negative stain electron microscopy images were acquired with a FEI Tecnai G2 transmission electron microscope and a TVIPS F416 CMOS camera.
Data analysis

- AFM data was analysed using custom written software in Matlab R2015b.
- Cortical fluorescence intensity data and actin regrowth experiments were analysed using Koretechs (http://www.matebio.com/software/koretechs) described in Biro et al, Cytoskeleton, 70:741-54, 2013.
- Western blot data were analysed in ImageJ v1.5.
- SEM data were analysed with a custom ImageJ plugin described in Chugh et al, Nature Cell Biology, 19:689-697, 2017.
- Cortical thickness data were analysed using custom written code Clark et al, Biophysical Journal, 105:570-580, 2013.
- TIRF images were analysed using ImageJ v1.5.
- Electron microscopy data were hand-picked using XMP3 software from the image processing framework SCIPION. Two- and Three-dimensional class averages were obtained with Relion v3.0.
- 3D reconstructions of protein complexes were fitted to crystal structures with UCSF Chimera v1.14.
- Statistical analysis and normality tests were performed in Excel 2013, Matlab R2015b and R v3.5.2.
- All other analysis was conducted using custom-written code in Matlab R2015b.

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☐ Life sciences  ☐ Behavioural & social sciences  ☐ Ecological, evolutionary & environmental sciences

For a reference copy of the document with all sections, see nature.com/documents/nr-reportsummary-faq.pdf

Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size

For each experiment, we estimated the required sample size based on expected change in the parameter of interest. After performing the experiments, the observed statistical power (i.e. post-hoc power) was also calculated.

Data exclusions

Where analysis involved curve fitting, goodness of fit was determined using the coefficient of determination, R-squared, and curves with R-squared<0.8 were excluded from statistical analysis.

For measurements on cells, for each dataset, outliers were defined as the values that fell outside the range [q1-w(q3-q1),q3+w(q3-q1)], where q1 and q3 were the 25th and 75th percentiles of the data and w was 1.5. Outliers were excluded from statistical analysis. The reported n numbers in the manuscript are the number of data points included in the statistical analysis after data exclusion. Criteria for data exclusion were pre-established.

Replication

For experiments on cells, all replicates reported in the manuscript are biological replicates collected from experiments performed on at least 3 individual days.

Our in vitro, single filament experiments were each repeated at least twice, on different days. Replicates are consistent with the data but are not shown. The data shown are those from the day with the highest statistics (the largest number of analyzed filaments).

Randomization

On each day of experiment, care was taken to perform control and perturbation conditions in a randomised order.

Blinding

No blinding was performed. Unbiased analysis of data was carried out where possible.

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.
Antibodies

**Antibodies used**

This information is indicated in the following format:

Protein, Manufacturer, Catalog number, clone, dilution used for Western blotting (WB) or immunostaining (IF), host species.

**Primary antibodies:**
- GAPDH, Novus Biological, NB300-221, clone 1D4, WB: 1:10000, Mouse
- mH2a, Abcam, ab96784, no clone id available, WB: 1:200, Rabbit
- Arp2, Santa Cruz, sc-10125, no clone id available, WB: 1:100, Goat
- Arp3, Cell Signalling, #4738, no clone id available, WB: 1:200, Rabbit
- IQGAP1, Sigma-Aldrich, WH0008826M1, clone 2C5, WB: 1:200, IF:100, Mouse
- SPIN90, Abcam, ab88467, no clone id available, WB: 1:500, IF: 1:100, Mouse
- SPIN90, Santa Cruz, sc-514232, no clone id available, IF: 1:50, Mouse
- NAP1/L Cook, Abcam, ab96715, no clone id available, WB: 1:500, IF: 1:100, Rabbit
- NAP1/L Cook, Abcam, ab156016, no clone id available, WB: 1:1000, IF: 1:100, Rabbit
- Flightless-I, Abcam, ab108594, no clone id available, WB: 1:1000, Rabbit
- Phospho-MLC, Cell Signalling, #3675, no clone id available, IF: 1:100, Mouse
- WASP2, gift from Dr Alexis Gautreau (Ecole Polytechnique, Palaiseau, France), IF: 1:50, Rabbit
- alpha-tubulin, Developmental Studies Hybridoma Bank, clone 12G10, WB: 1:1000, Mouse

**Secondary antibodies:**
- anti-mouse IgG (H+L) HRP, Sigma-Aldrich, GENA931-1000UL, WB:1:10,000, Sheep
- anti-mouse IgG (H+L) HRP, Jackson Immunoresearch, 115-035-003, WB: 1:10,000, Goat
- anti-rabbit IgG (H+L) HRP, Jackson Immunoresearch, 111-035-003, WB: 1:10,000, Goat
- anti-goat IgG (H+L) HRP, Jackson Immunoresearch, 705-035-003, WB: 1:10,000, Donkey
- anti-mouse IgG (H+L) Alexa 488, Invitrogen, A11001, IF: 1:200, Goat
- anti-mouse IgG (H+L) Alexa 568, Invitrogen, A11004, IF: 1:200, Goat
- anti-rabbit IgG (H+L) Alexa 488, Invitrogen, A11049, IF: 1:300, Goat
- anti-rabbit IgG (H+L) Alexa 568, Invitrogen, A11031, IF: 1:200, Goat

**Validation**

All antibodies utilized in this study are commercially obtained and were validated by the commercial suppliers (see specific websites for full information).

The WASP2 antibody was validated in Gautreau et al, PNAS, 101:4279-4283, 2004.

In addition, in our experiments, we verified that the size of the protein detected was the one expected.

Eukaryotic cell lines

**Policy information about cell lines**

**Cell line source(s)**

M2 melanoma blebbing cells were a gift of Prof Tom Stossel (Harvard Medical School, Boston, MA).

Wild-type HeLa cells were gift from the MPI-CBG Technology Development Studio (TDS).

HLK293T cells were acquired from ATCC (ATCC CRL-3216).

**Authentication**

None of the cell lines were authenticated.

**Mycoplasma contamination**

All lines were routinely screened for the presence of mycoplasma using the mycoALERT kit (Lonza). Cells used in this study tested negative for mycoplasma contamination.

**Commonly misidentified lines**

(See [ICLAC register](#).

No commonly misidentified cell lines were used.

Animals and other organisms

**Policy information about studies involving animals**

**ARRIVE guidelines** recommended for reporting animal research.

**Laboratory animals**

Adults female Xenopus laevis were used to obtain oocytes that were fertilised with sperm obtained from adult males. Embryos used in this study were at Xenopus stage 9.
| Study aspect                  | Description                                                                 |
|------------------------------|------------------------------------------------------------------------------|
| Wild animals                 | No wild animals were used in this study.                                    |
| Field-collected samples      | No field-collected samples were used in this study.                         |
| Ethics oversight             | All animal experiments complied with the Biological Service Unit at University College London and UK Home Office guidelines (Animal Act 1986). The protocols used in this study were approved by the UCL Animal Welfare and Ethical Review Body. |

Note that full information on the approval of the study protocol must also be provided in the manuscript.