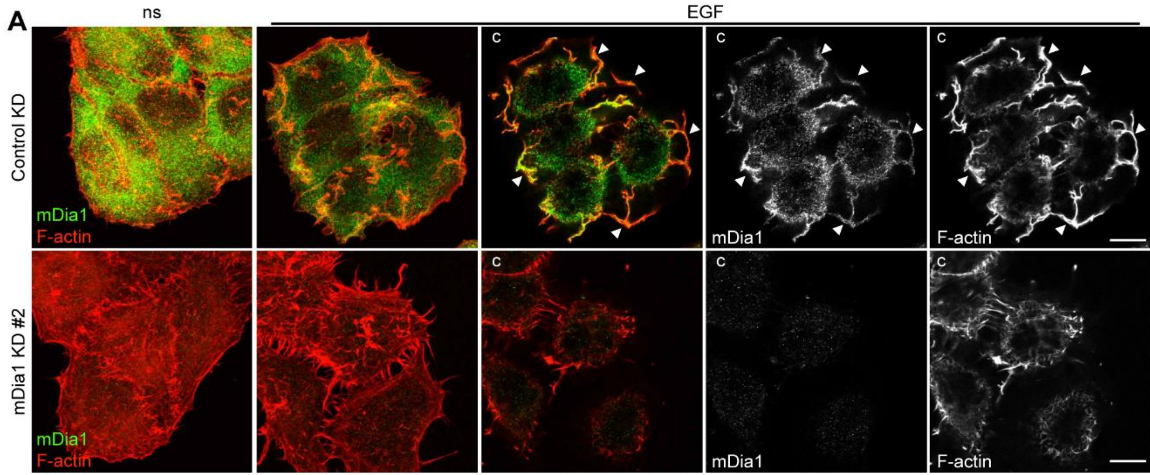
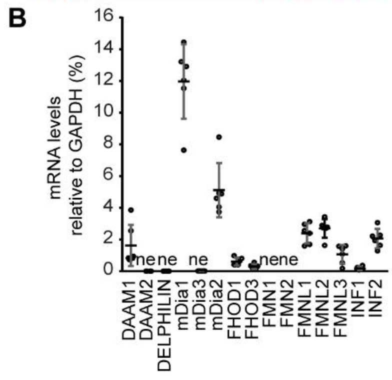
SUPPLEMENTAL FIGURES





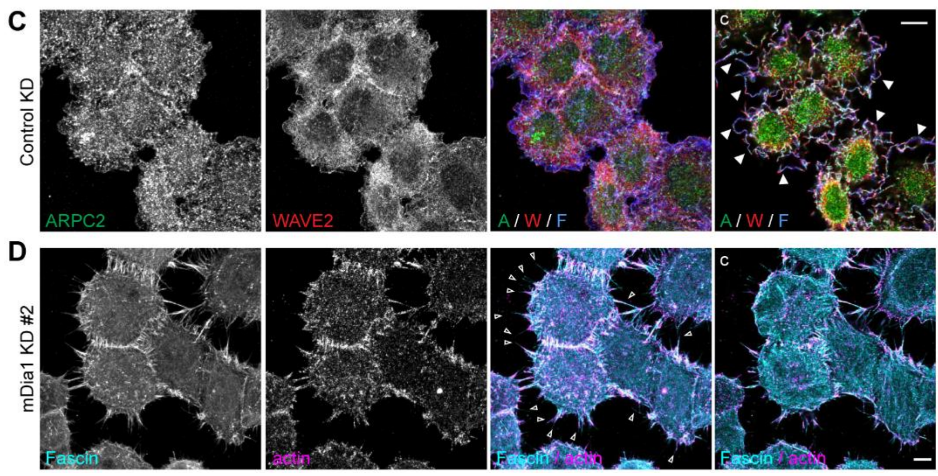


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Figure S1. Endogenous mDia1 localizes within EGF-induced ruffles and participates in the formation of ruffles and lamellipodia in multiple cell lines.

(A) Endogenous mDia1 localizes within EGF-induced ruffles. Control (Control KD) and mDia1 knockdown (mDia1 KD #2) HeLa cells were serum-starved (ns) and then either stimulated with EGF (EGF; 100 ng ml-1 for 7 minutes) or left untreated. Fixed cells were stained with anti-mDia1 antibodies (green in merge) and TRITC-phalloidin (red in merge). Representative maximal and central confocal sections (c) of EGF-treated cells are shown. The enrichment of endogenous mDia1 within expanding EGF-induced ruffles was confirmed using EGFP as volumetric marker, as shown in Fig. 3. Similar results were obtained using a different anti-mDia1 antibody (not shown). Note that the comparison between control and mDia1 KD cells demonstrates the specificity of the employed antibodies. White arrowheads mark ruffles. Bar, 10 µm. (B) Formin expression landscape in HeLa cells. Expression of Formins was assessed by RT-qPCR as explained in the Methods starting from total mRNA isolated from exponentially growing HeLa cells. Data are presented as mean ± s.d. of two independent mRNA isolations, each consisting of three technical replicates. *DAAM2, DELPHILIN, mDia3, FMN1* and *FMN2* are not expressed (ne). (C-D) Serum-starved and EGF-stimulated control (Control KD) and mDia1 knockdown (mDia1 KD #2) HeLa cells were fixed and stained with the indicated antibodies. (ARPC2 (A): green in merge; WAVE2 (W): red in merge, and F-actin (F): blue in merge). Representative maximal projections or central confocal sections (c) are presented. As cells were fixed in ice-cold Methanol to enable detection of Fascin with anti-Fascin antibodies (Fascin: false-coloured cyan in merge), anti-actin antibodies were used to label the actin cytoskeleton (actin: false-coloured magenta in merge). Bar, 10 µm.

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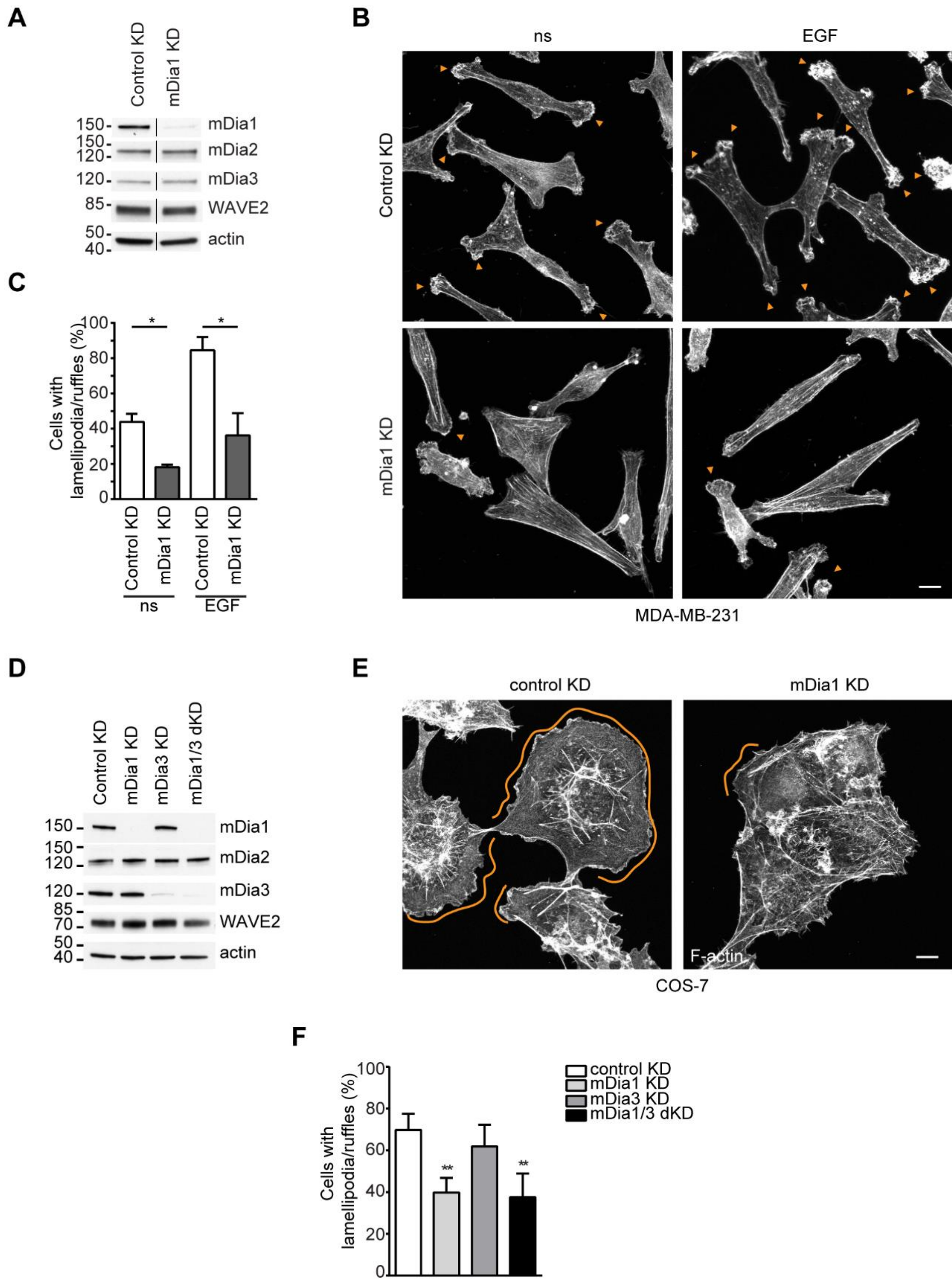

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Figure S2. mDia1 participates in the formation of ruffles and lamellipodia also in MDA-MB-231 and COS-7 cells.

(A) Stable control (Control KD) and mDia1 knockdown (mDia1 KD) MDA-MB-231 cells were characterized with the indicated antibodies. One of two experiments that were performed with similar results is shown. (B) Stable control (Control KD) and mDia1 knockdown (mDia1 KD) MDA-MB-231 were plated on collagen-coated coverslips, serum-starved overnight and then either stimulated with EGF (25 ng ml-1) for 7 minutes (EGF) or left untreated (ns). After fixation, cells were stained with TRITC-phalloidin. Representative maximal projections are presented. Orange arrowheads mark ruffles. Bar, 10 µm. (C) Percentage of ruffling cells was quantified for both serum-starved and EGF-stimulated cells. Graph depicts mean ± s.d. (t-test; * = $p < 0.05$; n = 170-177 cells from two independent experiments). (D) Stable control knockdown (Control KD) and mDia1 knockdown (mDia1 KD), mDia3 knockdown (mDia3 KD) or mDia1/mDia3 double knockdown (mDia1/3 dKD) COS-7 cells were characterized with the indicated antibodies. One of two experiments that were performed with similar results is shown. (E) Control and mDia1 knockdown COS-7 cells were plated on fibronectin-coated coverslips, kept in low serum and stained with TRITC-phalloidin. Representative maximal projections are shown. Areas with lamellipodia/ruffles are surrounded by orange lines. Bar, 10 µm. (F) Percentage of ruffling cells was quantified and plotted as mean ± s.d. (One-way ANOVA (Bonferroni's Multiple Comparison Test); ** = $p < 0.01$; n = 171-206 cells from three independent experiments).

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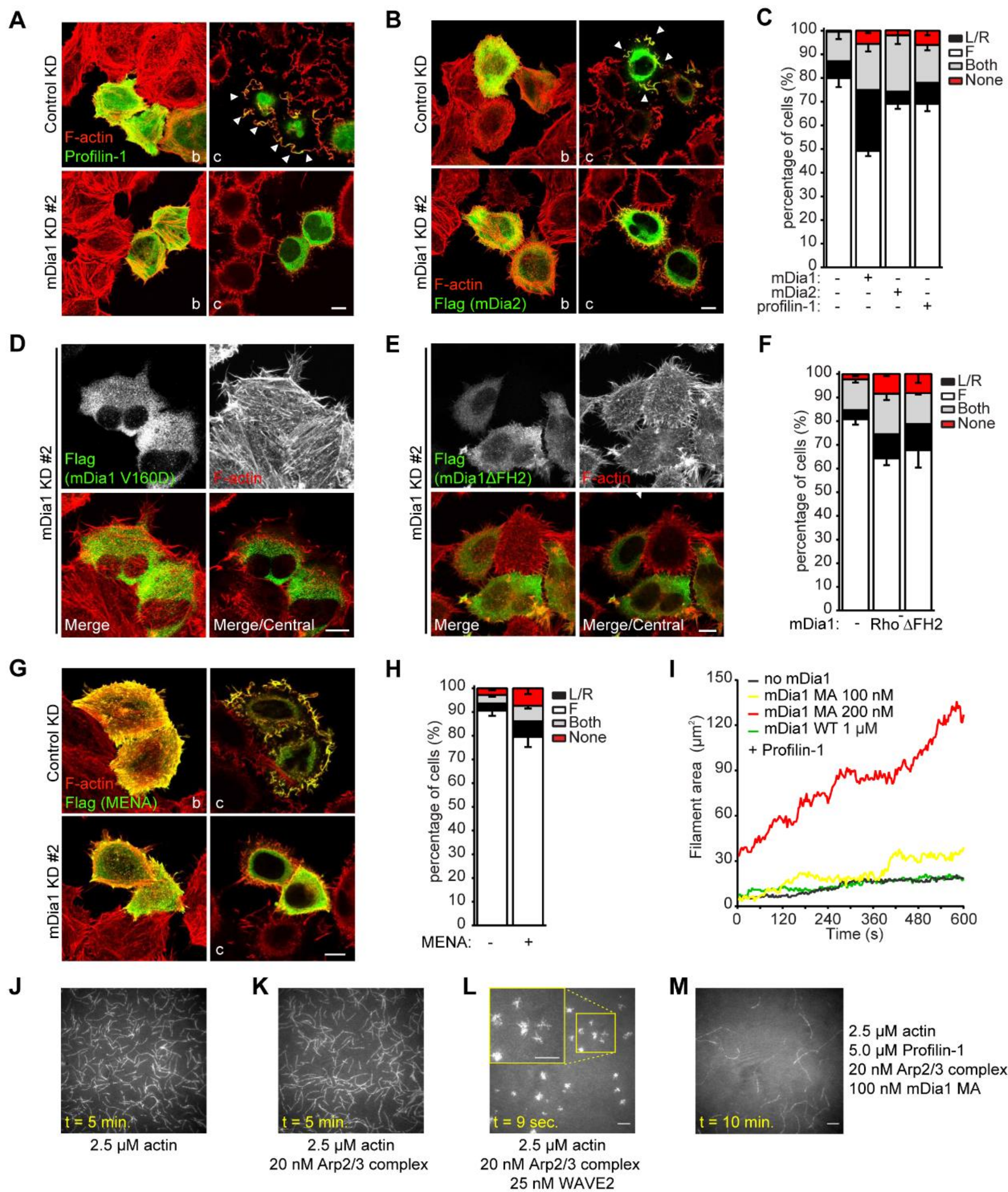

Figure S3. Profilin-1, mDia2, mDia1ΔFH2, mDia1 V160D and Mena did not rescue lamellipodium/ruffle formation in mDia1 KD cells, and mDia1 is an auto-inhibited actin nucleator and does not activate the Arp2/3 complex directly.

(A-C) Overexpression of Profilin-1 and mDia2 fail to rescue lamellipodia/ruffle formation in mDia1 KD cells. Control KD and mDia1 KD #2 cells were transfected with either EGFP-tagged Profilin-1 (A) or Flag-tagged mDia2 (B), serum-starved and stimulated with EGF. After fixation,

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exogenous mDia2 was detected using anti-Flag antibodies (green in merge). TRITC-phalloidin (red in merge) was used to detect actin filaments. Representative basal (b) and central confocal sections (c) are shown. White arrowheads mark ruffles. (C) EGF-induced protrusions formed by mDia1 KD cells from (B-C) were quantified as in Fig. 1E. Graph shows mean ± s.e.m. (One-WAY ANOVA; n = 306-308 cells from three independent experiments). Supplementary Material Table S4 shows statistical analysis of these experiments. (D-F) The Rho-binding and the FH2 domain of mDia1 are required for EGF-induced lamellipodium/ruffle formation. mDia1 KD #2 cells were transfected with Flag-tagged mDia1 V160D (D) or Flag-tagged mDia1ΔFH2 (E), and processed as indicated above. (F) EGF-induced protrusions of cells from (D-E) were quantified as in Fig. 1E. (Rho^- = mDia1 V160D; ΔFH2 = mDia1ΔFH2). Graph shows mean ± s.e.m. (One-WAY ANOVA; n = 306-308 cells from three independent experiments). Supplementary Material Table S5 shows statistical analysis of these experiments. (G-H) Mena does not rescue ruffle formation in mDia1 KD cells. mDia1 KD #2 cells were transfected with Flag-tagged Mena and processed as indicated above. (H) EGF-induced protrusions formed by Mena-transfected cells from (G) were quantified as in Fig. 1E. Graph shows mean ± s.e.m. (One-WAY ANOVA; n > 200 cells from two independent experiments). Supplementary Material Table S6 shows statistical analysis of these experiments. All bars, 10 µm. (I) mDia1 MA is an auto-inhibited actin nucleator. Actin (2.5 µM) was polymerized in the presence of Profilin-1 either alone or in with the indicated concentrations of mDia1 WT or mDia1 MA. Area filled with actin filaments was quantified using representative movies as described in Materials and Methods. (J-L) The Arp2/3 complex is unable to form branches in the absence of WAVE2. Actin was polymerized either alone (I) or in the presence of the Arp2/3 complexes with or without WAVE2 (K and L, respectively). Conditions are indicated below the micrographs (t = time; min. = minutes; sec. = seconds). Bars, 10 µm. (M) mDia1 does not activate the Arp2/3 complex directly. Actin was polymerized in the presence of Profilin-1, the Arp2/3 complex and mDia1 MA, but without WAVE2. Conditions are indicated beside the micrograph (t = time; min. = minutes). Bar 10 µm.

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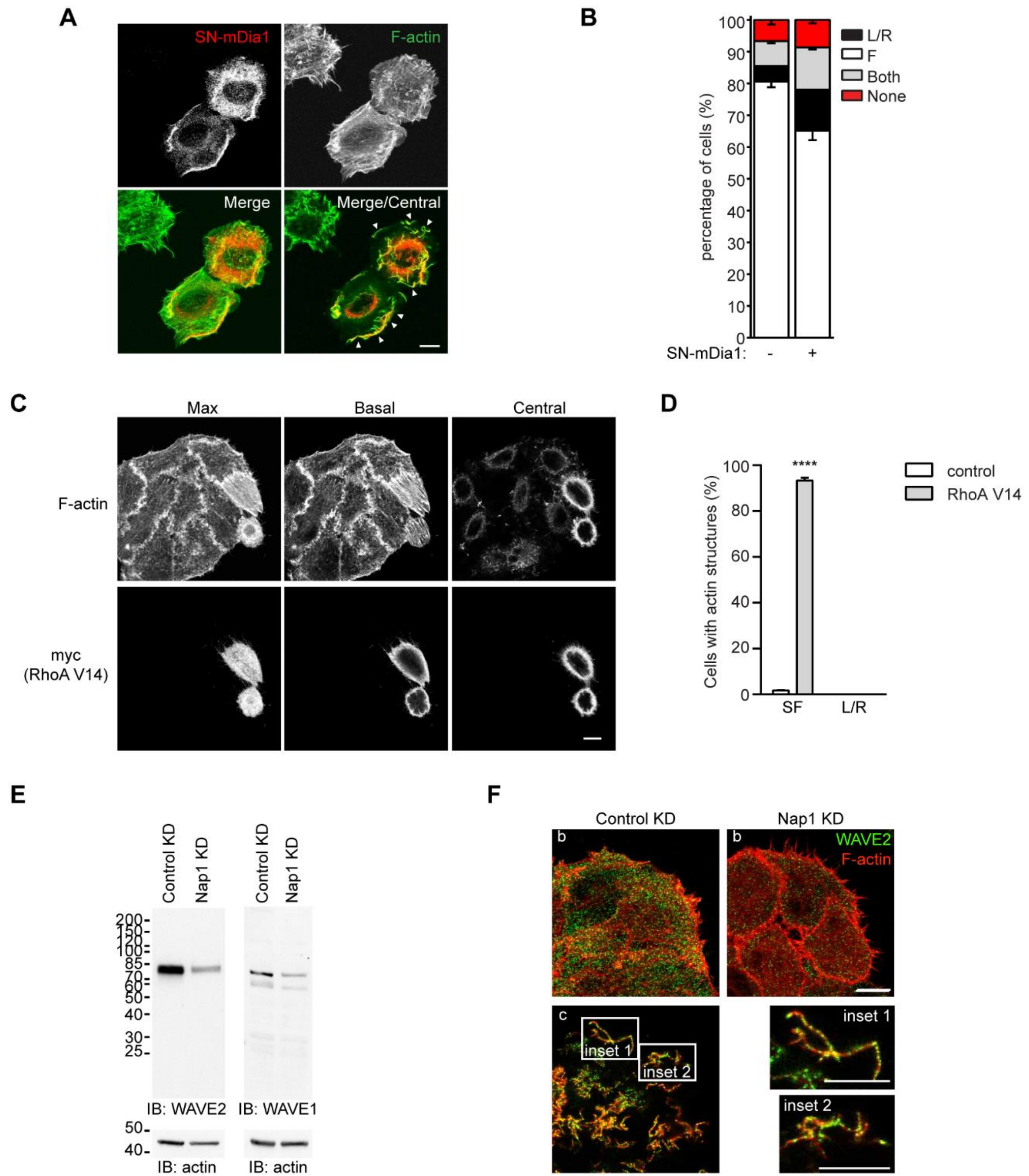

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Figure S4. SuperNova-mDia1 rescues EGF-induced ruffling in mDia1 KD cells, constitutively active Rho is insufficient to induce lamellipodia/ruffles, and characterization of anti-WAVE2 antibodies.

(A-B) mDia1 KD #2 cells were transfected with SuperNova-mDia1 (SN-mDia1), serum-starved and stimulated with EGF (100 ng ml^{-1}; 7 minutes). After fixation, cells were stained with anti-KillerRed antibodies (red in merge) and FITC-phalloidin (green in merge) to detect SuperNova-mDia1 and actin filaments, respectively. Representative maximal projections and central confocal sections (Merge/Central) are shown. White arrowheads mark lamellipodia and ruffles. Bar, 10 µm. B: EGF-induced protrusions of cells from (A) were quantified as in Fig. 1E. Graph shows mean ± s.e.m. (One-WAY ANOVA; $n \geq 400$ cells from three independent experiments). Supplementary Material Table S7 shows statistical analysis of these experiments. (C) Wild-type HeLa cells were transfected with myc-tagged constitutively active RhoA (RhoA V14), serum starved and fixed. After fixation, cells were stained with anti-myc antibodies and TRITC-phalloidin to detect RhoA V14 and actin filaments, respectively. Representative maximal projections and central confocal sections (Merge/Central) are shown. Bar, 10 µm. (D) Cells from (A) were quantified for increased stress-fiber (SF) formation and lamellipodia/ruffle (L/R) formation. Graph shows mean ± s.e.m. (t-test; **** = $p < 0.0001$; $n \geq 300$ cells from three independent experiments). No lamellipodia/ruffles were observed in three independent experiments. (E) Specificity of anti-WAVE2 antibodies. Total cell lysates obtained from control and Nap1 knockdown cells were blotted with anti-WAVE2, anti WAVE1 and anti-actin antibodies. Note that the anti-WAVE2 antibodies detect a single species whose intensity decreases upon silencing of Nap1. Consistent with the downregulation of the WAVE complex in Nap1 KD cells, the expression of WAVE1 was also reduced in these cells. (F) Specificity of anti-WAVE2 antibodies and localization of endogenous WAVE2. Control and Nap1 knockdown cells were stimulated with EGF (100 ng ml^{-1}) for 7 minutes, processed and stained with anti-WAVE2 antibodies (green in the merge) and TRITC-Phalloidin (red in the merge). Basal (b) and central confocal sections (c) of EGF-treated cells are shown. Insets (1 and 2) zoom in to the localization of endogenous WAVE2 within two different ruffling area. Bars, 10 µm.

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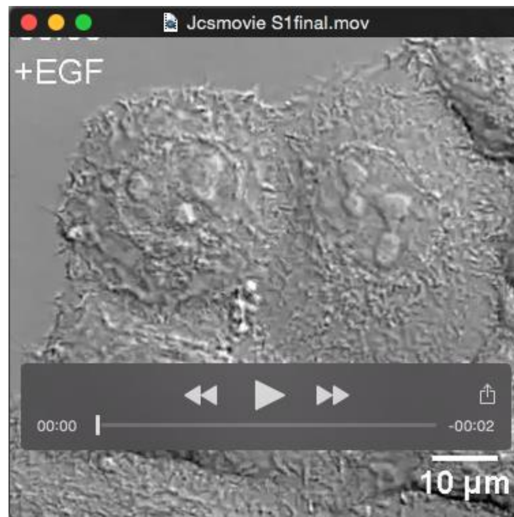
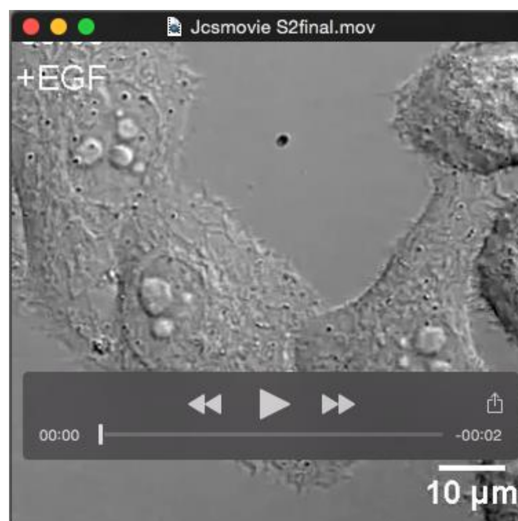
SUPPLEMENTAL MOVIES



Movie 1

Control HeLa cells form dynamic lamellipodium-like and ruffle-like protrusions upon EGF stimulation. Images were acquired every 15 seconds.



Movie 2

mDia1 KD #2 HeLa cells form dynamic filopodium-like protrusions growing by extension upon EGF stimulation. Images were acquired every 15 seconds and acquired in parallel to Movie 1.

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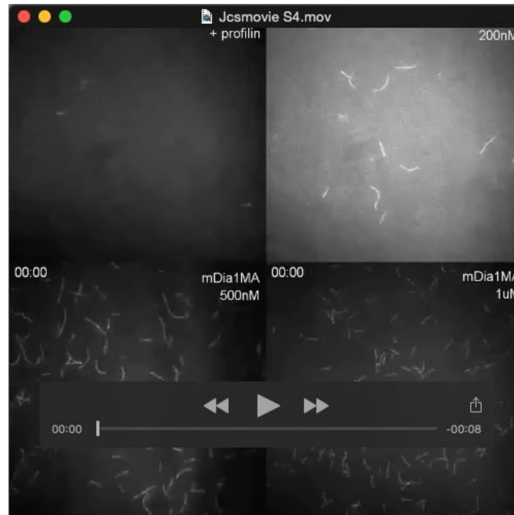
Movie 3

mDia1 WT is an auto-inhibited Formin. Profilin-actin was polymerized in the absence or presence of 1 μM of mDia1 WT. Imaging was carried out as described in the Materials and Methods.



Movie 4

mDia1 MA nucleates and elongates actin filaments in the presence of Profilin-1. Profilin-bound actin was polymerized in the absence or presence of indicated concentration of mDia1 MA. Imaging was carried out as described in the Materials and Methods.

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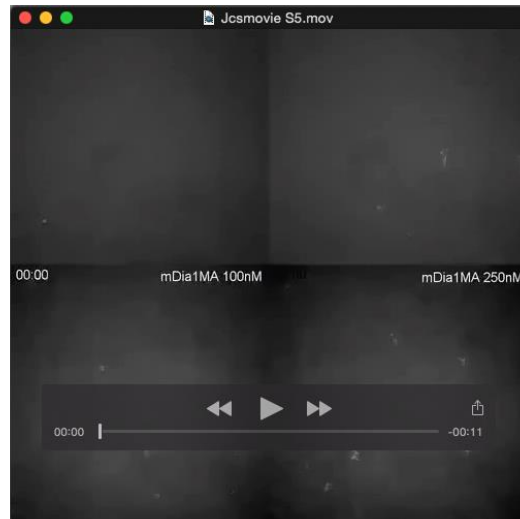

Movie 5

mDia1 polymerizes linear actin filaments activating the Arp2/3 complex. A reaction containing profilin-actin, full-length 25 nM WAVE2 and 20 nM Arp2/3 complex was polymerized in the absence or presence of indicated concentration of mDia1 MA. Imaging was carried out as described in the Materials and Methods.

Movie 6

Demonstration of the effectiveness of Chromophore-Assisted Laser Inactivation (CALI) of SuperNova-mDia1. mDia1 knockdown #2 HeLa cells were transiently transfected with SuperNova-mDia1 and serum starved overnight. Chromophore-Assisted Laser Inactivation (CALI) was performed prior to addition of EGF (100 ng ml^{-1}). Images were acquired every 5 seconds. Arrows highlight lamellipodia/ruffles. Note that CALI of SN-mDia1 prevented ruffling. Bar, 10 μm.

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Movie 7

mDia1 is dispensable for expansion of lamellipodia/ruffles. mDia1 knockdown #2 HeLa cells were transiently transfected with SuperNova-mDia1, serum starved overnight and stimulated 3 minutes with EGF (100 ng ml^{-1}) prior to Chromophore-Assisted Laser Inactivation (CALI). Images were acquired every 5 seconds. Arrows highlight lamellipodia/ruffles. Note that CALI of SN-mDia1 did not stop ruffling. Bar, 10 µm.

Table S1 - Statistical analysis corresponding to Figure 1E

Bonferroni's multiple comparisons test	Mean Difference	Significant?	Summary	Adj. P Value
Filopodia				
shControl *vs.* shDIAPH1 #1	-51.9	Yes	****	< 0,0001
shControl *vs.* shDIAPH1 #2	-75.6	Yes	****	< 0,0001
Ruffling/Lamellipodia				
shControl *vs.* shDIAPH1 #1	74.8	Yes	****	< 0,0001
shControl *vs.* shDIAPH1 #2	81.17	Yes	****	< 0,0001
Both				
shControl *vs.* shDIAPH1 #1	-14.4	Yes	***	0.0007
shControl *vs.* shDIAPH1 #2	2.233	No	ns	> 0,9999
None				
shControl *vs.* shDIAPH1 #1	-8.567	Yes	*	0.0422
shControl *vs.* shDIAPH1 #2	-7.733	No	ns	0.071

One-WAY ANOVA (Bonferroni's Multiple Comparison Test; * = $p < 0.05$; *** = $p < 0.001$; **** = $p < 0.0001$; $n \geq 300$ cells from three independent experiments).

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Table S2 - Statistical analysis corresponding to Figure 2B

Bonferroni's multiple comparisons test	Mean Difference	Significant?	Summary	Adj. P Value
Filopodia				
shDIAPH1 #1 *vs.* shDIAPH1 #1 + mDia1	20.73	Yes	**	0.0025
shDIAPH1 #2 *vs.* shDIAPH1 #2 + mDia1	27.17	Yes	***	0.0002
Ruffling/Lamellipodia				
shDIAPH1 #1 *vs.* shDIAPH1 #1 + mDia1	-19.50	Yes	**	0.0042
shDIAPH1 #2 *vs.* shDIAPH1 #2 + mDia1	-17.73	Yes	*	0.0207
Both				
shDIAPH1 #1 *vs.* shDIAPH1 #1 + mDia1	-2.000	No	ns	> 0.9999
shDIAPH1 #2 vs. shDIAPH1 #2 + mDia1	-7.733	No	ns	> 0.9999
None				
shDIAPH1 #1 *vs.* shDIAPH1 #1 + mDia1	0.7667	No	ns	> 0.9999
shDIAPH1 #2 *vs.* shDIAPH1 #2 + mDia1	-1.767	No	ns	> 0.9999

One-WAY ANOVA (Bonferroni's Multiple Comparison Test; * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$; $n \geq 300$ cells from three independent experiments).

Table S3 - Statistical analysis of membrane protrusions formed by control KD HeLa cells overexpressing mDia1 compared to non-transfected ones

Bonferroni's multiple comparisons test	Mean Difference	Significant?	Summary	Adj. P Value
Filopodia	-1.283	No	ns	0.9819
Ruffling/Lamellipodia	7.075	No	ns	0.0909
Both	-1.108	No	ns	0.9895
None	-4.675	No	ns	0.3603

One-WAY ANOVA (Bonferroni's Multiple Comparison Test; * = $p < 0.05$; **** = $p < 0.0001$; $n \geq 200$ cells from four independent experiments).

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Table S4 - Statistical analysis corresponding to Figure S3C

Bonferroni's multiple comparisons test	Mean Difference	Significant?	Summary	Adj. P Value
Filopodia				
shDIAPH1 #2 *vs.* shDIAPH1 #2 + mDia1	30.80	Yes	****	< 0.0001
shDIAPH1 #2 *vs.* shDIAPH1 #2 + mDia2	10.90	Yes	*	0.0124
shDIAPH1 #2 *vs.* shDIAPH1 #2 + Profilin-1	10.90	Yes	*	0.0124
Ruffling/Lamellipodia				
shDIAPH1 #2 *vs.* shDIAPH1 #2 + mDia1	-18.60	Yes	****	< 0.0001
shDIAPH1 #2 *vs.* shDIAPH1 #2 + mDia2	1.967	No	ns	> 0.9999
shDIAPH1 #2 *vs.* shDIAPH1 #2 + Profilin-1	-1.733	No	ns	> 0.9999
Both				
shDIAPH1 #2 *vs.* shDIAPH1 #2 + mDia1	-7.000	No	ns	0.1678
shDIAPH1 #2 *vs.* shDIAPH1 #2 + mDia2	-11.33	Yes	**	0.0090
shDIAPH1 #2 *vs.* shDIAPH1 #2 + Profilin-1	-3.500	No	ns	0.9862
None				
shDIAPH1 #2 *vs.* shDIAPH1 #2 + mDia1	-5.200	No	ns	0.4511
shDIAPH1 #2 *vs.* shDIAPH1 #2 + mDia2	-1.667	No	ns	> 0.9999
shDIAPH1 #2 *vs.* shDIAPH1 #2 + Profilin-1	-5.600	No	ns	0.3671

One-WAY ANOVA (Bonferroni's Multiple Comparison Test; * = $p < 0.05$; ** = $p < 0.01$; **** = $p < 0.0001$; $n \geq 300$ cells from three independent experiments).

Table S5 - Statistical analysis corresponding to Figure S3F

Bonferroni's multiple comparisons test	Mean Difference	Significant?	Summary	Adj. P Value
Filopodia				
shDIAPH1 #2 *vs.* shDIAPH1 #2 + V160D-mDia1	13.22	Yes	***	0.0008
shDIAPH1 #2 *vs.* shDIAPH1 #2 + mDia1ΔFH2	16.65	Yes	****	< 0.0001
Ruffling/Lamellipodia				
shDIAPH1 #2 vs. shDIAPH1 #2 + V160D-mDia1	-7.183	No	ns	0.0834
shDIAPH1 #2 *vs.* shDIAPH1 #2 + mDia1ΔFH2	-6.283	No	ns	0.1459
Both				
shDIAPH1 #2 vs. shDIAPH1 #2 + V160D-mDia1	-0.06667	No	ns	> 0.9999
shDIAPH1 #2 *vs.* shDIAPH1 #2 + mDia1ΔFH2	-4.200	No	ns	0.4498
None				
shDIAPH1 #2 vs. shDIAPH1 #2 + V160D-mDia1	-5.967	No	ns	0.1758
shDIAPH1 #2 *vs.* shDIAPH1 #2 + mDia1ΔFH2	-6.167	No	ns	0.1563

One-WAY ANOVA (Bonferroni's Multiple Comparison Test; * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$; $n \geq 300$ cells from three independent experiments).

Table S6 - Statistical analysis corresponding to Figure S3H

Bonferroni's multiple comparisons test	Mean Difference	Significant?	Summary	Adj. P Value
Filopodia	11.07	Yes	**	0.0061
Ruffling/Lamellipodia	-3.567	No	ns	0.9457
Both	-2.900	No	ns	> 0.9999
None	-4.600	No	ns	0.5287

One-WAY ANOVA (Bonferroni's Multiple Comparison Test; * = $p < 0.05$; **** = $p < 0.0001$; $n \geq 300$ cells from three independent experiments).

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Table S7 - Statistical analysis corresponding to Figure S4B

Bonferroni's multiple comparisons test	Mean Difference	Significant?	Summary	Adj. P Value
Filopodia	15.43	Yes	****	< 0.0001
Ruffling/Lamellipodia	-8.025	Yes	*	0.0104
Both	-5.400	No	ns	0.1324
None	-2.050	No	ns	> 0.9999

One-WAY ANOVA (Bonferroni's Multiple Comparison Test; * = $p < 0.05$; **** = $p < 0.0001$; $n \geq 400$ cells from four independent experiments).

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Table S8 - Summary of optogenetic manipulation of SuperNova-mDia1

Percentage of SN-mDia1 expressing cells that underwent CALI prior to EGF stimulation and ruffled	Percentage of SN-mDia1 expressing cells that ruffled upon EGF stimulation	Percentage of SN-mDia1 expressing cells that underwent CALI after EGF stimulation and ruffled
13.8% (5/36)	26.2% (17/63)	28.1% (16 / 57)

Summary of the CALI experiments showing percentage of SuperNova-mDia1-expressing cells that formed EGF-induced lamellipodia/ruffles, as assessed by live-imaging. Data represents cells imaged from three to four independent experiments imaged on different days. Note that percentage of SN-mDia1-expressiong cells subjected to CALI prior to EGF stimulation is similar to the percentage of ruffling observed in Fig. 1E, thereby showing the effective inactivation of SN-mDia1.

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Table S9 - Statistical analysis corresponding to Figure 6E

Bonferroni's multiple comparisons test	Mean Difference	Significant?	Summary	Adj. P Value
Filopodia	19.1	Yes	****	< 0,0001
Ruffling/Lamellipodia	-5.067	Yes	*	0.0235
Both	-9.433	Yes	****	< 0,0001
None	-4.6	Yes	*	0.0433

One-WAY ANOVA (Bonferroni's Multiple Comparison Test; * = $p < 0.05$; **** = $p < 0.0001$; $n \geq 150$ cells from three independent experiments).

Table S10 - Primer sequences used for PCR amplification

Construct name	Primer 1 (5'-3')	Primer 2 (5'-3')
Profilin-1	CGGGATCCGCCGGGTGGAACGCCTAC	CGGAATTCTCAGTACTGGGAACGCCG
V160D mDia1	GTCCCTTCGAGACTCTCTCAACAATAA	TCAAGGCAGCTAAGCAGGTG
mDia1 MA	GACAGGTGTGGCGGACAGTCTTC	GAAGACTGTCCGCCACACCTGTC
mDIa1ΔFH2	GAGGGGGATGAGACAGGTG	GGTTAATCCAAATGGCAGAACTGG
SN-mDia1	AACCGGTCGCCACCATGGGTTCAGAGG	AAAGATCTGAGTCCGGAATCCTCGTC GCTACCGATGGC