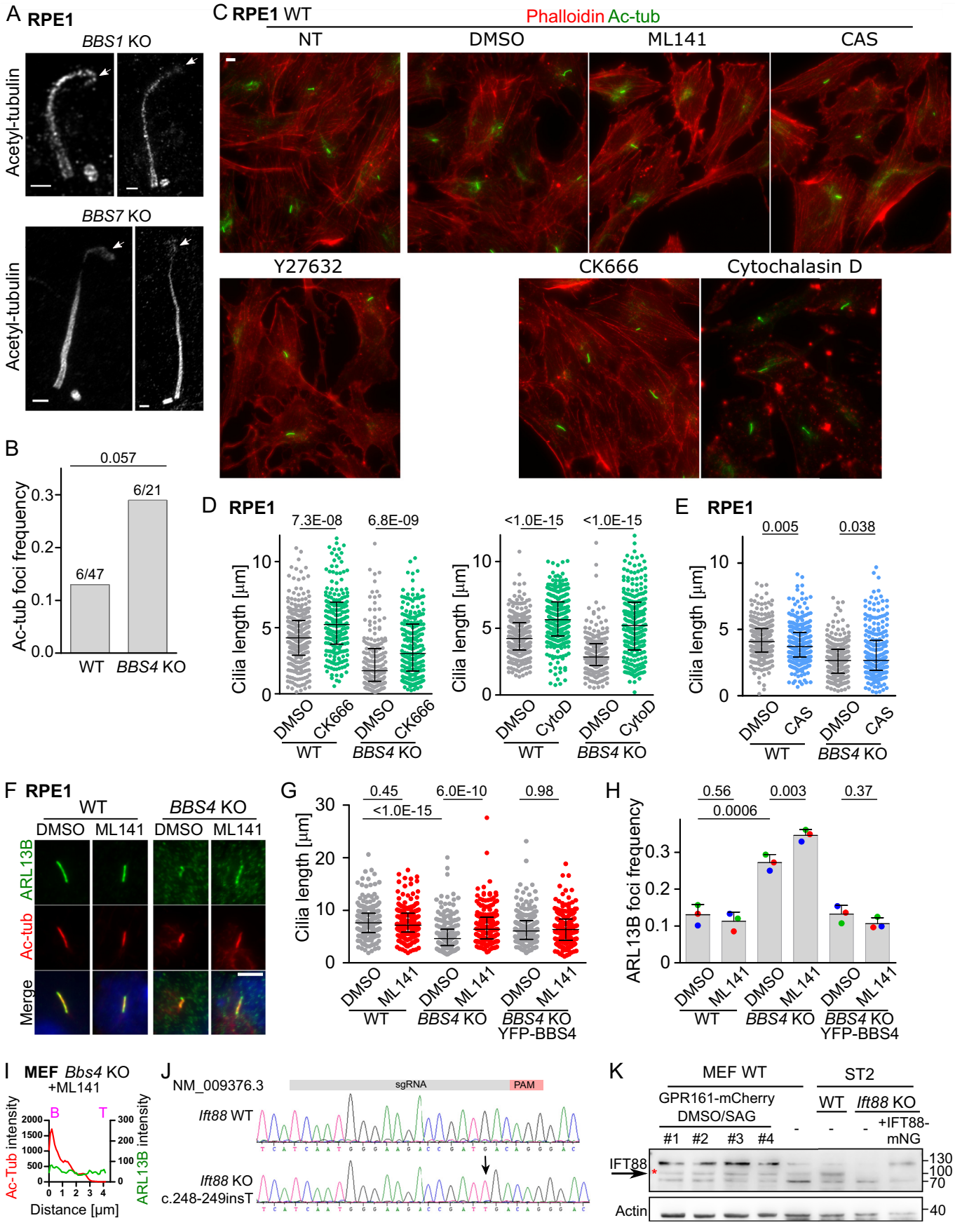
Expanded View Figures

Figure EV1. Actin regulators regulate the cilia length in different manner.

(**A**) Expansion microscopy of the cilia axoneme of the *BBS1*$^{KO/KO}$ and *BBS7*$^{KO/KO}$ RPE1 cells visualized by staining with antibody to acetylated tubulin. White arrows point to bulges at the cilia tips. Scale bar, 2 µm. (**B**) The graph shows the frequency of bulges at the cilia tips visualized by expansion microscopy of cilia in WT and *BBS4*$^{KO/KO}$ RPE1 cells. The number of observed bulges out of the total count of imaged cilia is shown. Statistical analysis was done using the contingency table and one-sided Chi-square test. (**C**) Representative micrographs of the cilia visualized by staining with antibody to acetylated tubulin (Ac-tub) and staining of actin cytoskeleton with Phalloidin-TexasRed in the WT RPE1 cells treated with inhibitors of ROCK1/RHOA (Y27632), CDC42 (ML141), RAC1 (CAS1177865-17-6 (CAS)), ARP2/3 (CK666), and actin polymerization (Cytochalasin D) for 2 h. Scale bar, 5 µm. (**D**) Quantification of the cilia length based on acetylated tubulin signal in the WT and *BBS4*$^{KO/KO}$ RPE1 cells treated with DMSO or CK666 or Cytochalasin D (CytoD) for 2 h as in (**C**). Medians with interquartile range from three independent experiments ($n = 220–320$ cilia). (**E**) Quantification of the cilia length based on acetylated tubulin signal in the WT and *BBS4*$^{KO/KO}$ RPE1 cells treated with vehicle or CAS for 2 h as in (**C**). Medians with interquartile range from three independent experiments ($n = 250–280$ cilia). (**F**) Representative micrographs of the cilia visualized by staining with antibody to acetylated tubulin (Ac-tub) and ARL13B in the WT and *BBS4*$^{KO/KO}$ RPE1 cells treated with DMSO or ML141 for 2 h. Scale bar, 5 µm. (**G**) Quantification of the cilia length in WT, *BBS4*$^{KO/KO}$, and *BBS4*$^{KO/KO}$ RPE1 cells expressing YFP-BBS4 treated with DMSO or ML141 for 2 h and stained with antibodies to acetylated tubulin (Ac-tub) and ARL13B. Medians with interquartile range from three independent experiments ($n = 200–250$ cilia). (**H**) Quantification of the frequency of ARL13B foci at the cilia tips in WT, *BBS4*$^{KO/KO}$, and *BBS4*$^{KO/KO}$ RPE1 cells expressing YFP-BBS4 treated with DMSO or ML141 for 2 h in (**G**). Mean and SD of three independent experiments ($n = 200–250$ cilia). (**I**) Intensity plot profiles of the Ac-tub and ARL13B signal measured from the cilia base (B) to the cilia tip (T) in *Bbs4* KO MEFs treated with ML141 in Fig. 1I. The ARL13B signal extends (~0.5 µm) beyond the Ac-tub signal. (**J**) Representative chromatogram analysis of DNA sequencing data for WT ST2 and *Ift88* KO ST2 cell clones reveals a homozygous mutation characterized by the insertion of a T nucleotide between bases 248 and 249 of the *Ift88* gene (NM_009376.3, arrow). The position of the sgRNA and PAM sequence used for targeting is indicated. (**K**) Representative Western blot from total cell lysates prepared from WT MEFs, SAG/DMSO treated GPR161-mCherry WT MEFs (experiments #1–4 in Fig. 1K–M), WT and *Ift88* KO ST2 cells and *Ift88* KO ST2 cells expressing the IFT88-mNeonGreen and probed with antibodies to IFT88 and β-actin. The arrow highlights the IFT88 band (red star) corresponding to the expected size of 100 kDa, which is absent in the *Ift88* KO ST2 cells. Data information: Statistical significance was calculated using the one-sided Chi-square test (**B**), two-tailed Mann–Whitney test (**D**, **E**, **G**) and two-tailed paired t-test (**H**) and the obtained *p*-values are indicated. Merged micrographs show nuclei staining with DAPI—blue (**F**).


A RPE1
BBS1 KO
BBS7 KO
Acetyl-tubulin
C RPE1 WT
Phalloidin Ac-tub
NT
DMSO
ML141
CAS
Y27632
CK666
Cytochalasin D
B
0.057
6/21
6/47
Ac-tub foci frequency
WT
BBS4 KO
D RPE1
7.3E-08
6.8E-09
<1.0E-15
<1.0E-15
Cilia length [µm]
DMSO
CK666
CytoD
E RPE1
0.005
0.038
CAS
F RPE1
WT
BBS4 KO
ARL13B
Ac-tub
Merge
G
0.45
<1.0E-15
6.0E-10
0.98
ML141
YFP-BBS4
H
0.56
0.0006
0.003
0.37
ARL13B foci frequency
I MEF Bbs4 KO
+ML141
Ac-Tub intensity
ARL13B intensity
Distance [µm]
J
NM_009376.3
sgRNA
PAM
Ift88 WT
Ift88 KO
c.248-249insT
K
MEF WT
ST2
GPR161-mCherry
DMSO/SAG
#1 #2 #3 #4
WT
Ift88 KO
+IFT88-mNG
IFT88
Actin

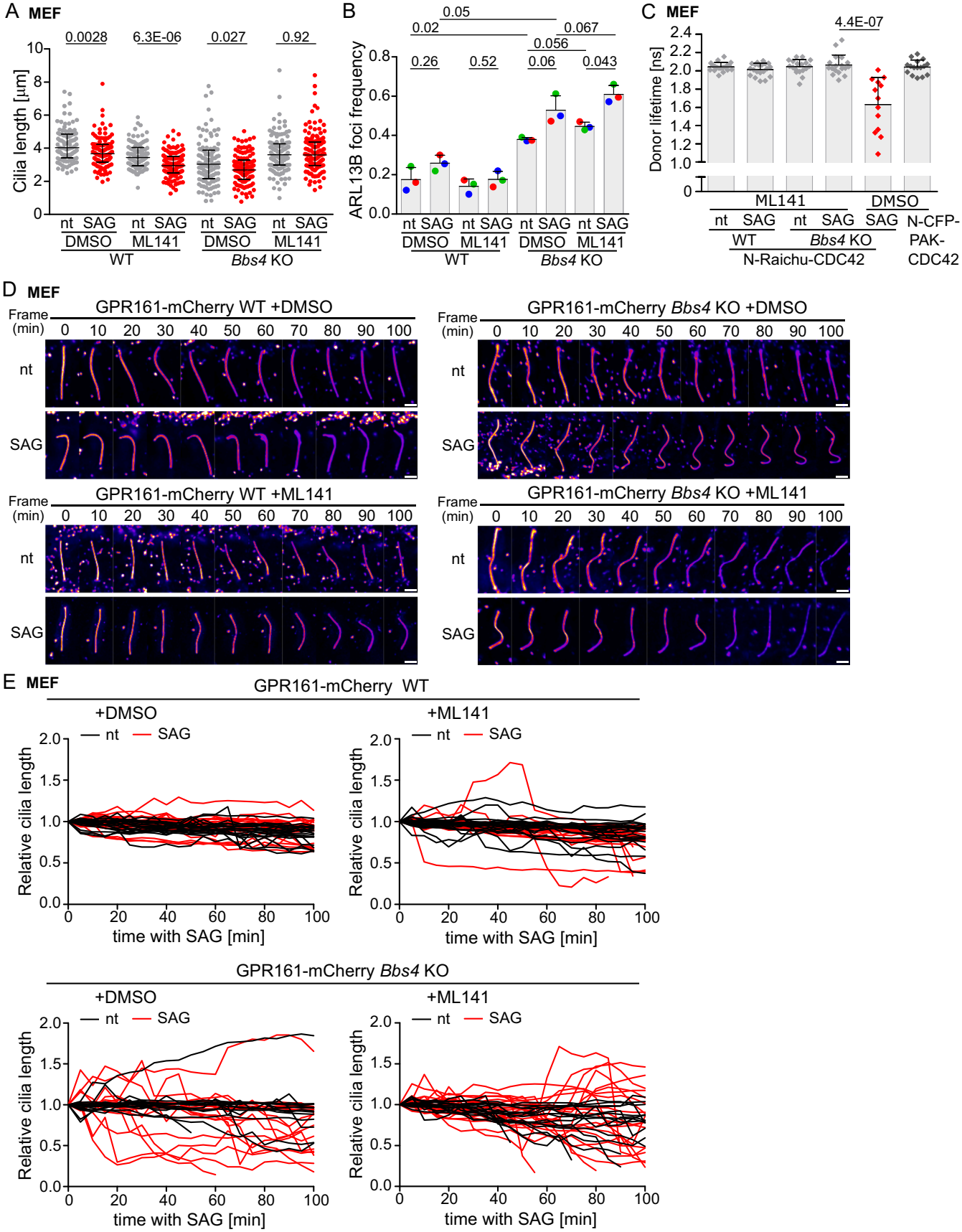

A MEF
0.0028
6.3E-06
0.027
0.92
Cilia length [µm]
nt SAG nt SAG nt SAG nt SAG
DMSO ML141 DMSO ML141
WT
Bbs4 KO
B
0.05
0.02
0.067
0.056
0.26
0.52
0.06
0.043
ARL13B foci frequency
nt SAG nt SAG nt SAG nt SAG
DMSO ML141 DMSO ML141
WT
Bbs4 KO
C MEF
4.4E-07
Donor lifetime [ns]
ML141
DMSO
nt SAG nt SAG SAG
WT
Bbs4 KO
N-Raichu-CDC42
N-CFP-PAK-CDC42
D MEF
GPR161-mCherry WT +DMSO
GPR161-mCherry Bbs4 KO +DMSO
GPR161-mCherry WT +ML141
GPR161-mCherry Bbs4 KO +ML141
Frame (min)
0 10 20 30 40 50 60 70 80 90 100
nt
SAG
E MEF
GPR161-mCherry WT
GPR161-mCherry Bbs4 KO
+DMSO
+ML141
nt
SAG
Relative cilia length
time with SAG [min]

Figure EV2. CDC42 hyperactivation drives cilia shortening in BBSome-deficient cells upon SHH triggering.

(**A**) Quantification of the cilia length, in non-treated (nt) and SAG induced—2 h, WT and *Bbs4*$^{KO/KO}$ MEFs concomitantly treated with DMSO or ML141 and stained with antibodies to acetylated tubulin (Ac-tub) and ARL13B. Medians with interquartile range from three independent experiments ($n = 90$–130 cilia). (**B**) Quantification of the frequency of ARL13B foci at the cilia tips in non-treated (nt) and SAG induced—2 h, WT and *Bbs4*$^{KO/KO}$ MEFs concomitantly treated with DMSO or ML141 in (**A**). Mean and SD of three independent experiments ($n = 90$–130 cilia). (**C**) The donor lifetime values extracted from the FRET-FLIM analysis of cilia as a region of interest in non-treated (nt) and SAG induced—2 h, WT and *Bbs4*$^{KO/KO}$ MEFs expressing N-Raichu-CDC42 treated with ML141 or DMSO and values measured for the no-FRET control expressed in WT MEFs treated with DMSO. Mean and SD of three (WT) and four (KO, no-FRET) independent experiments ($n = 13$–20 cilia). (**D**) Representative micrographs of the cilia in non-treated (nt) and SAG induced WT and *Bbs4*$^{KO/KO}$ MEFs expressing GPR161-mCherry, pre-treated for 30 min with DMSO or ML141 and imaged every 1 min for 100 min. Frames were extracted from the live cell videos (17–30 cilia per condition in two independent experiments). Scale bar, 2 μm. (**E**) Plots depict the variable dynamics of the length of the individual cilia in non-treated (nt) and SAG induced WT and *Bbs4*$^{KO/KO}$ MEFs expressing GPR161-mCherry, pre-treated for 30 min with DMSO or ML141 and imaged every 1 min for 100 min in (**D**). The length of the cilium was normalized to the length measured at time 0 min. In total 17–30 cilia were monitored per condition in two independent experiments. Data information: Statistical significance was calculated using the two-tailed Mann–Whitney test (**A**, **C**) and two-tailed paired t-test (**B**) and the obtained *p*-values are indicated.

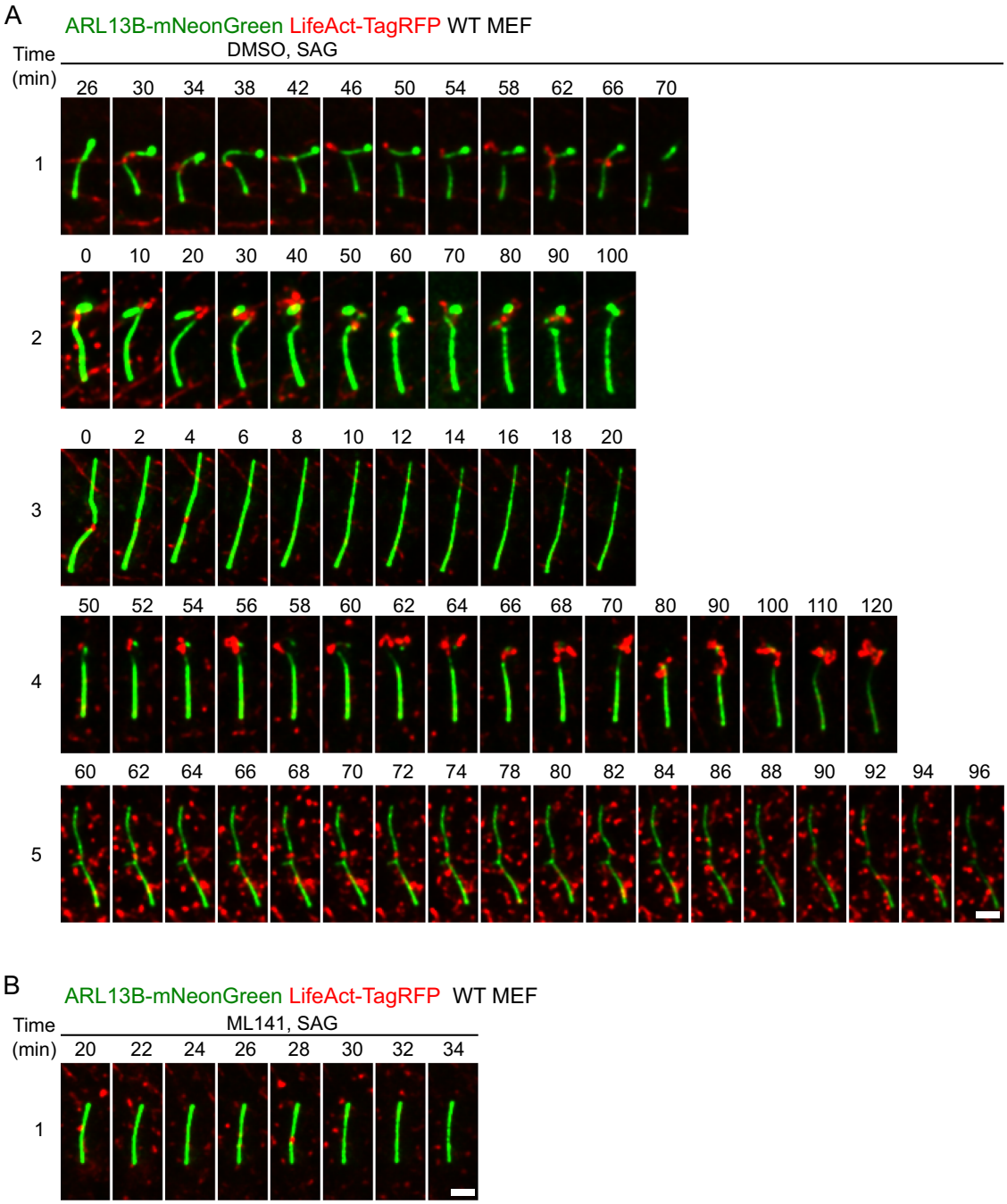


Figure EV3. CDC42 is required for actin polymerization in cilia in WT cells.

(**A**) Representative micrographs show F-actin polymerization events observed in cilia in WT MEFs expressing mNeonGreen-ARL13B (green) and LifeAct-TagRFP (red) treated with SAG and DMSO and imaged for 2 h. Frames were extracted from time-lapse videos (51 in total, 6 actin polymerization events, four independent experiments). Scale bar, 2 µm. (**B**) Representative micrographs show F-actin polymerization events observed in cilia in WT MEFs expressing mNeonGreen-ARL13B (green) and LifeAct-TagRFP (red) treated with SAG and ML141 and imaged for 2 h. Frames were extracted from time-lapse videos (54 in total, 2 actin polymerization events, four independent experiments). Scale bar, 2 µm. Maximum intensity projections of the z-stacks were done using Fiji ImageJ software and the intensities for both channels were adjusted post acquisition for better visualization.

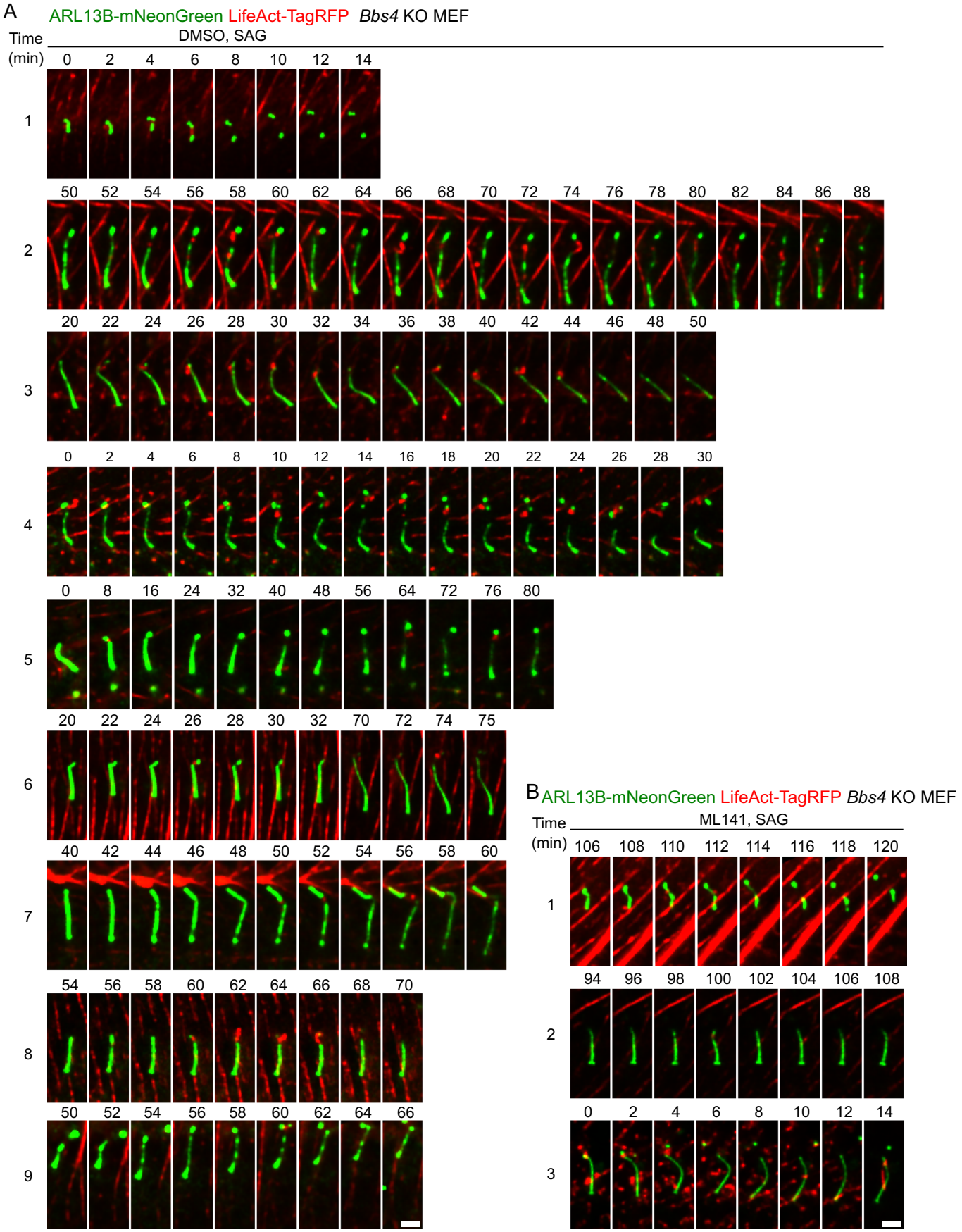

A
ARL13B-mNeonGreen LifeAct-TagRFP Bbs4 KO MEF
Time (min)
DMSO, SAG
0 2 4 6 8 10 12 14
1
50 52 54 56 58 60 62 64 66 68 70 72 74 76 78 80 82 84 86 88
2
20 22 24 26 28 30 32 34 36 38 40 42 44 46 48 50
3
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30
4
0 8 16 24 32 40 48 56 64 72 76 80
5
20 22 24 26 28 30 32 70 72 74 75
6
40 42 44 46 48 50 52 54 56 58 60
7
54 56 58 60 62 64 66 68 70
8
50 52 54 56 58 60 62 64 66
9
B
ARL13B-mNeonGreen LifeAct-TagRFP Bbs4 KO MEF
Time (min)
ML141, SAG
106 108 110 112 114 116 118 120
1
94 96 98 100 102 104 106 108
2
0 2 4 6 8 10 12 14
3

Figure EV4. CDC42 is required for actin polymerization in cilia in BBSome-deficient cells.

(**A**) Representative micrographs show F-actin polymerization events observed in cilia in *Bbs4*$^{KO/KO}$ MEFs expressing mNeonGreen-ARL13B (green) and LifeAct-TagRFP (red) treated with SAG and DMSO and imaged for 2 h. Frames were extracted from time-lapse videos (39 in total, 10 actin polymerization events, four independent experiments). Scale bar, 2 μm. (**B**) Representative micrographs show F-actin polymerization events observed in cilia in *Bbs4*$^{KO/KO}$ MEFs expressing mNeonGreen-ARL13B (green) and LifeAct-TagRFP (red) treated with SAG and ML141 and imaged for 2 h. Frames were extracted from time-lapse videos (39 in total, 4 actin polymerization events, four independent experiments). Scale bar, 2 μm. Maximum intensity projections of the z-stacks were done using Fiji ImageJ software and the intensities for both channels were adjusted post acquisition for better visualization.

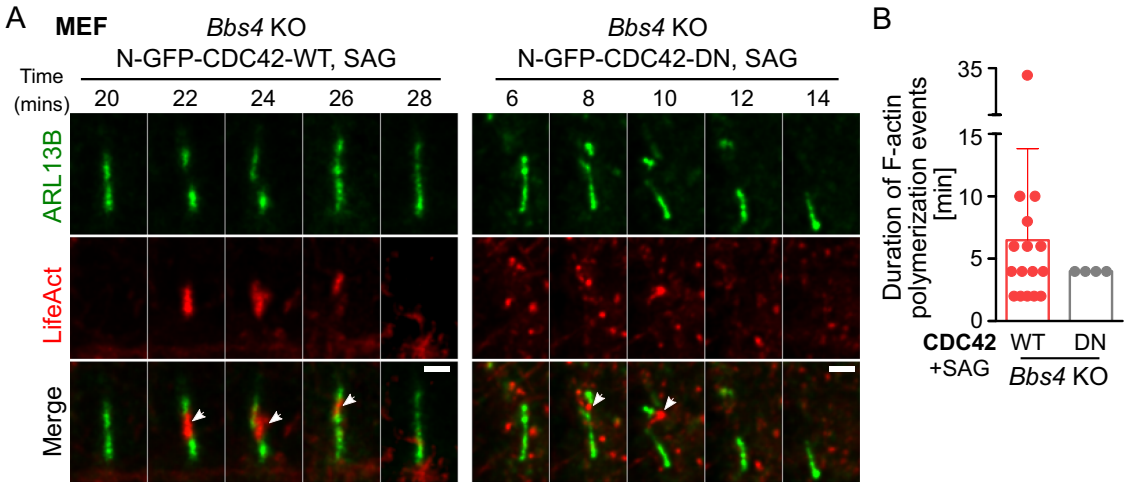


Figure EV5. Activity of cilia localized CDC42 is required for actin polymerization in cilia in BBSome-deficient cells.

(**A**) Representative micrographs depicting ciliary ectocytosis and actin polymerization detected by live cell imaging of *Bbs4*$^{KO/KO}$ MEFs expressing mNG-ARL13B and LifeAct-TagRFP in the presence of WT or DN version of CDC42. The cells were treated with SAG and imaged every 2 min for 2 h. White arrows point to the F-actin polymerization events. Cilia base at bottom. Scale bar, 2 μm. Maximum intensity projections of the z-stacks were done using Fiji ImageJ software and the intensities for both channels were adjusted post acquisition for better visualization. (**B**) The plot depicts the duration of the observed actin polymerization events detected in cilia in the *Bbs4*$^{KO/KO}$ MEFs expressing mNG-ARL13B and LifeAct-TagRFP in the presence of WT or DN version of CDC42 upon treatment with SAG during live cell imaging—six independent experiments in each condition (**A**). Means with SD are shown.