Supplementary Information

Small-molecule inhibitors of the AAA+ ATPase motor cytoplasmic dynein

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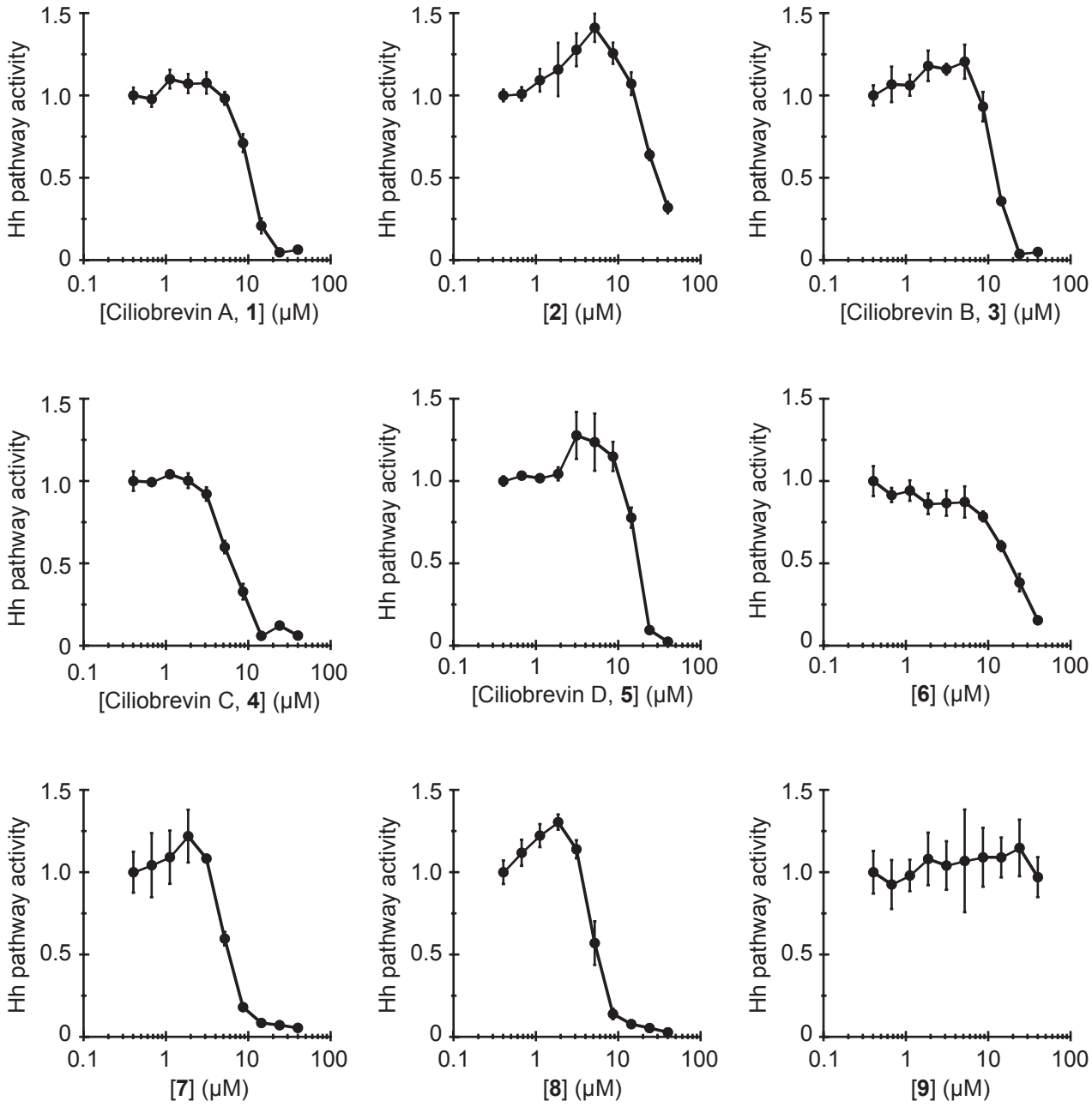
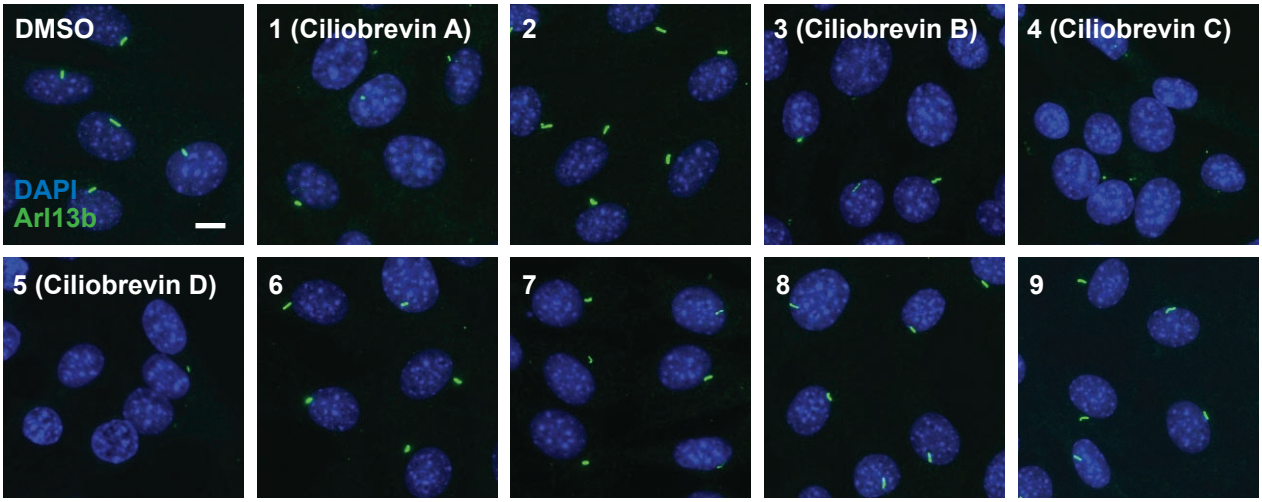


Supplementary Figure 1. Dose response of benzoyl dihydroquinazolinone analogs on Hh pathway activation. Compound effects on Hh pathway activity in Shh-LIGHT2 cells stimulated with Shh-N-conditioned medium. The cells were co-treated with doses of the indicated compounds for 24 hours. Data are the average of triplicate samples ± s.d.



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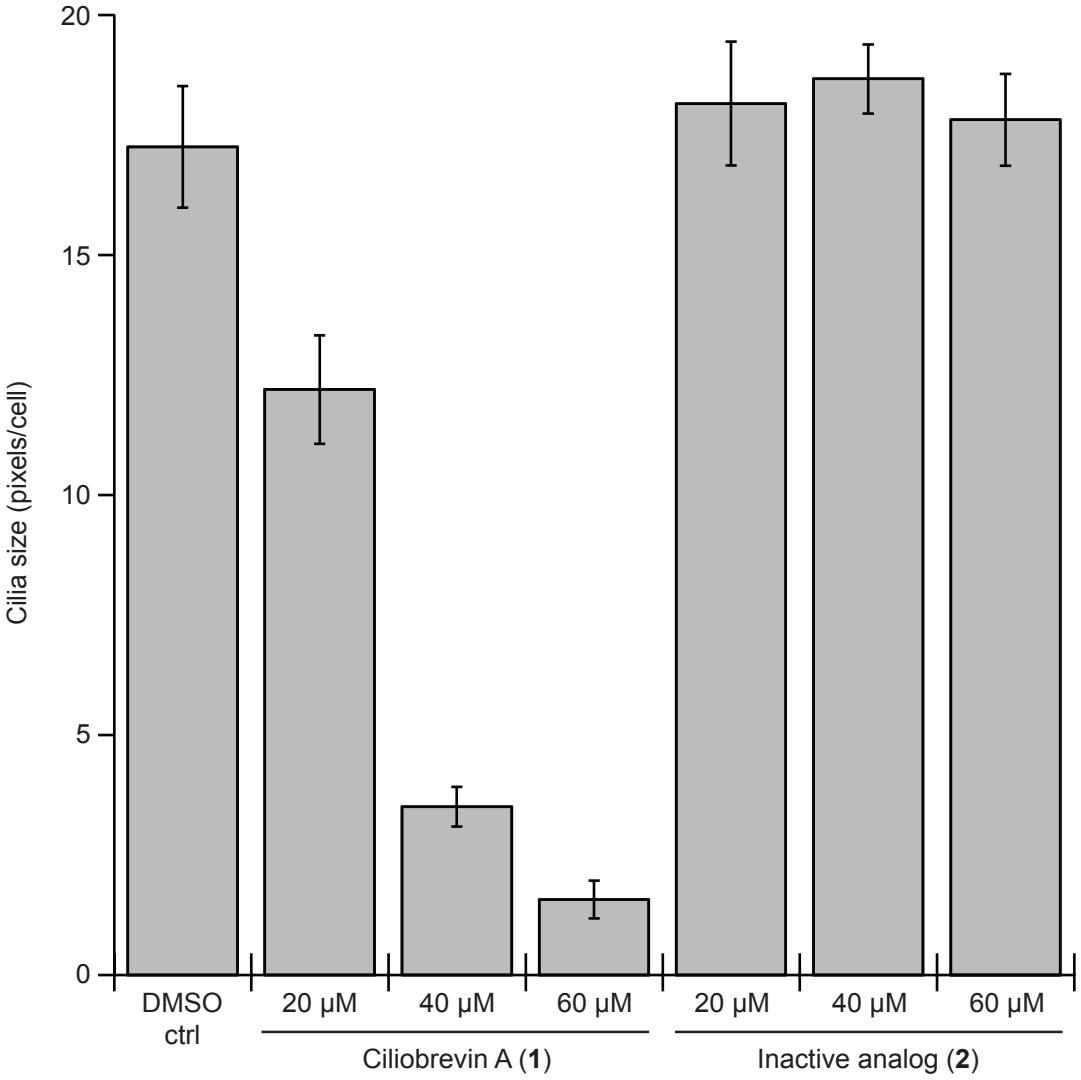

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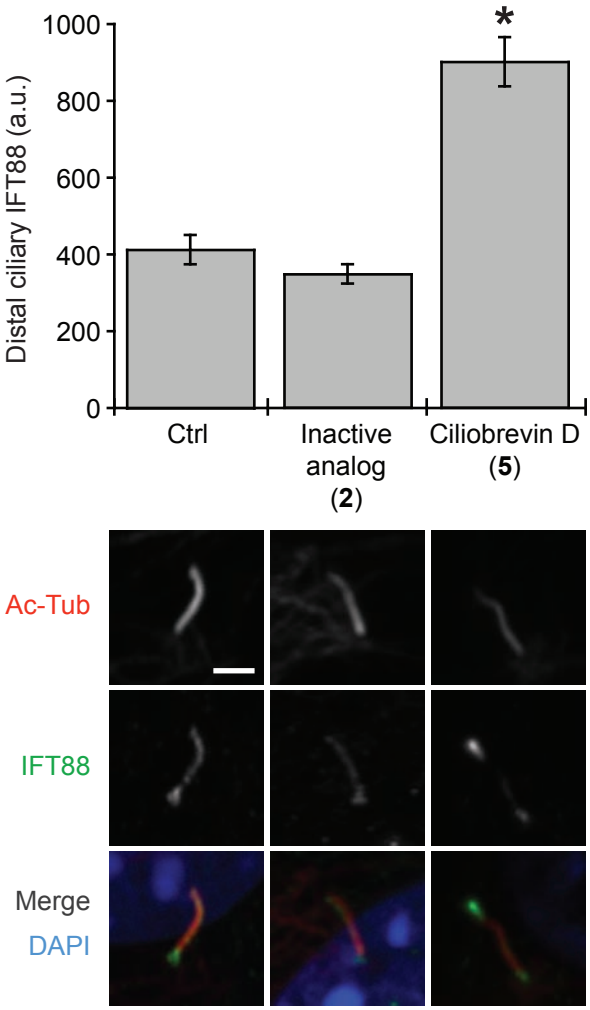

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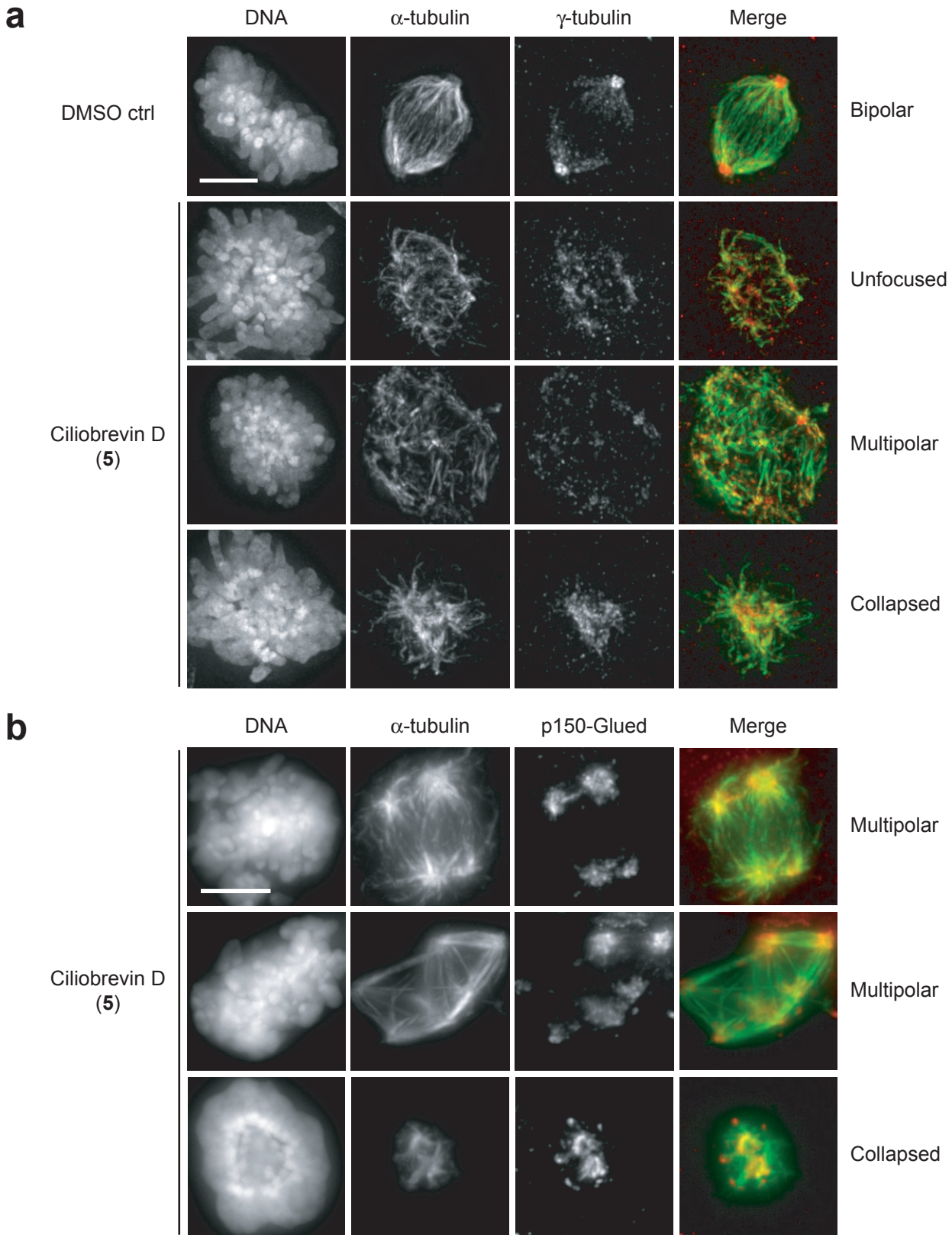

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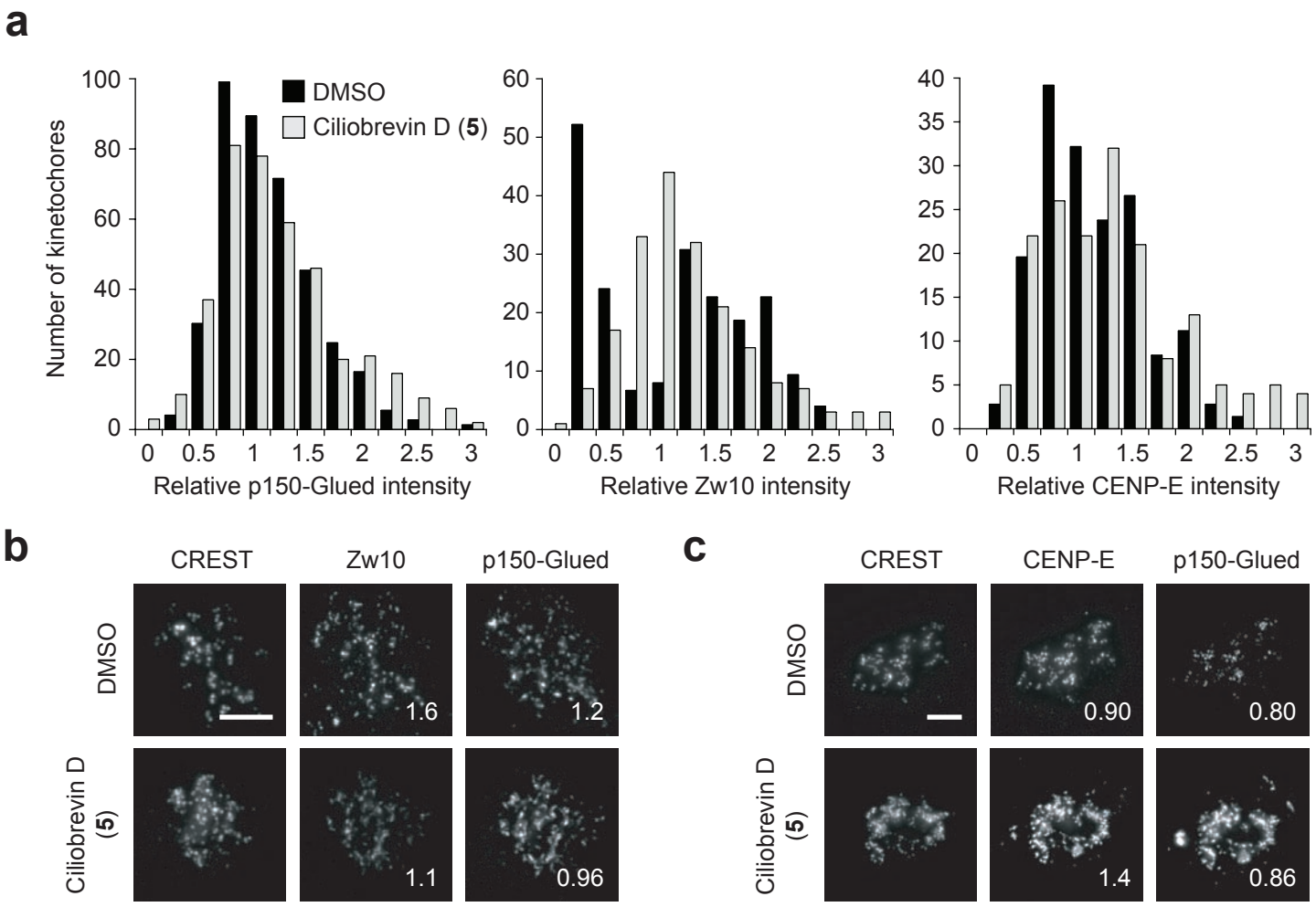

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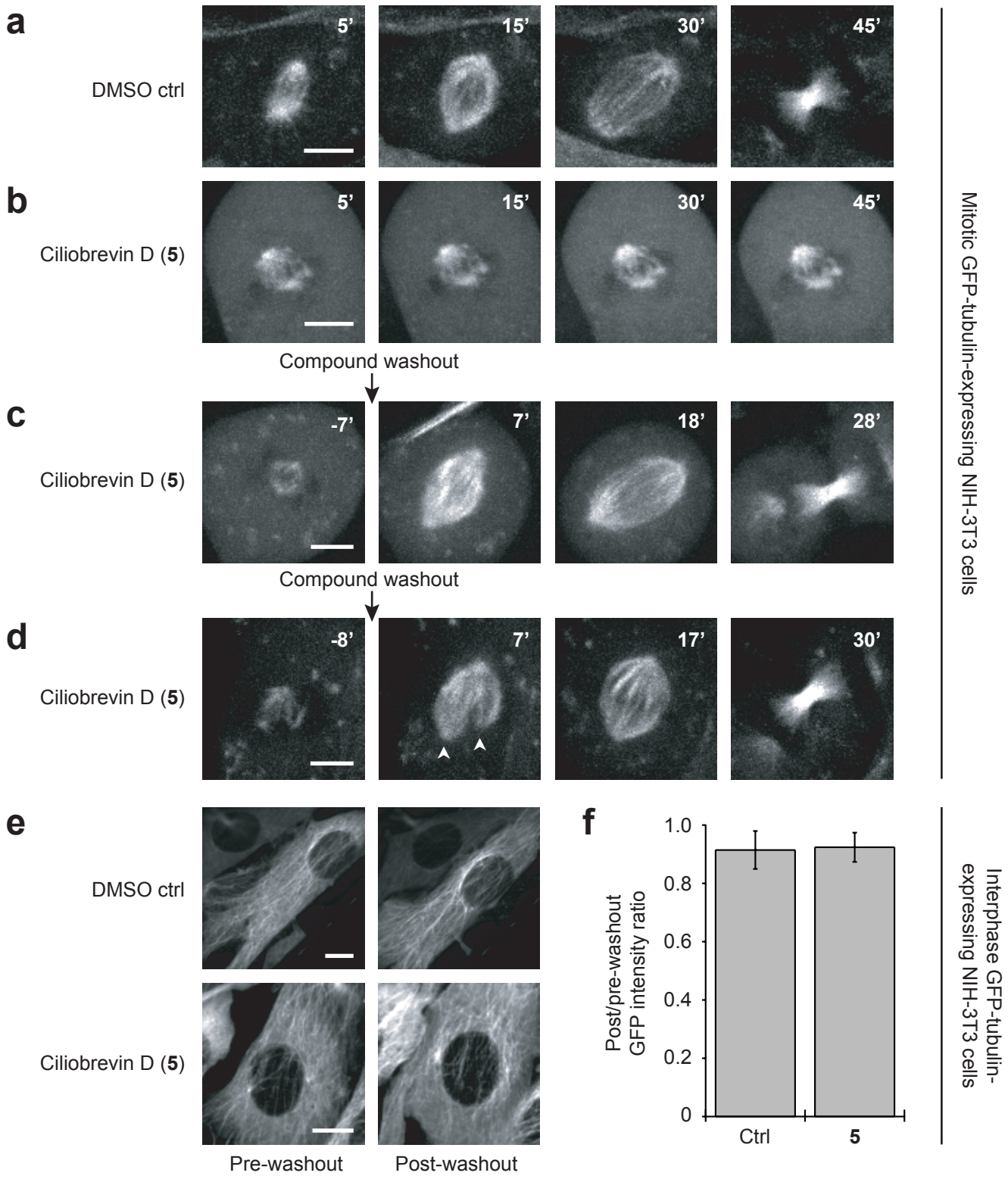

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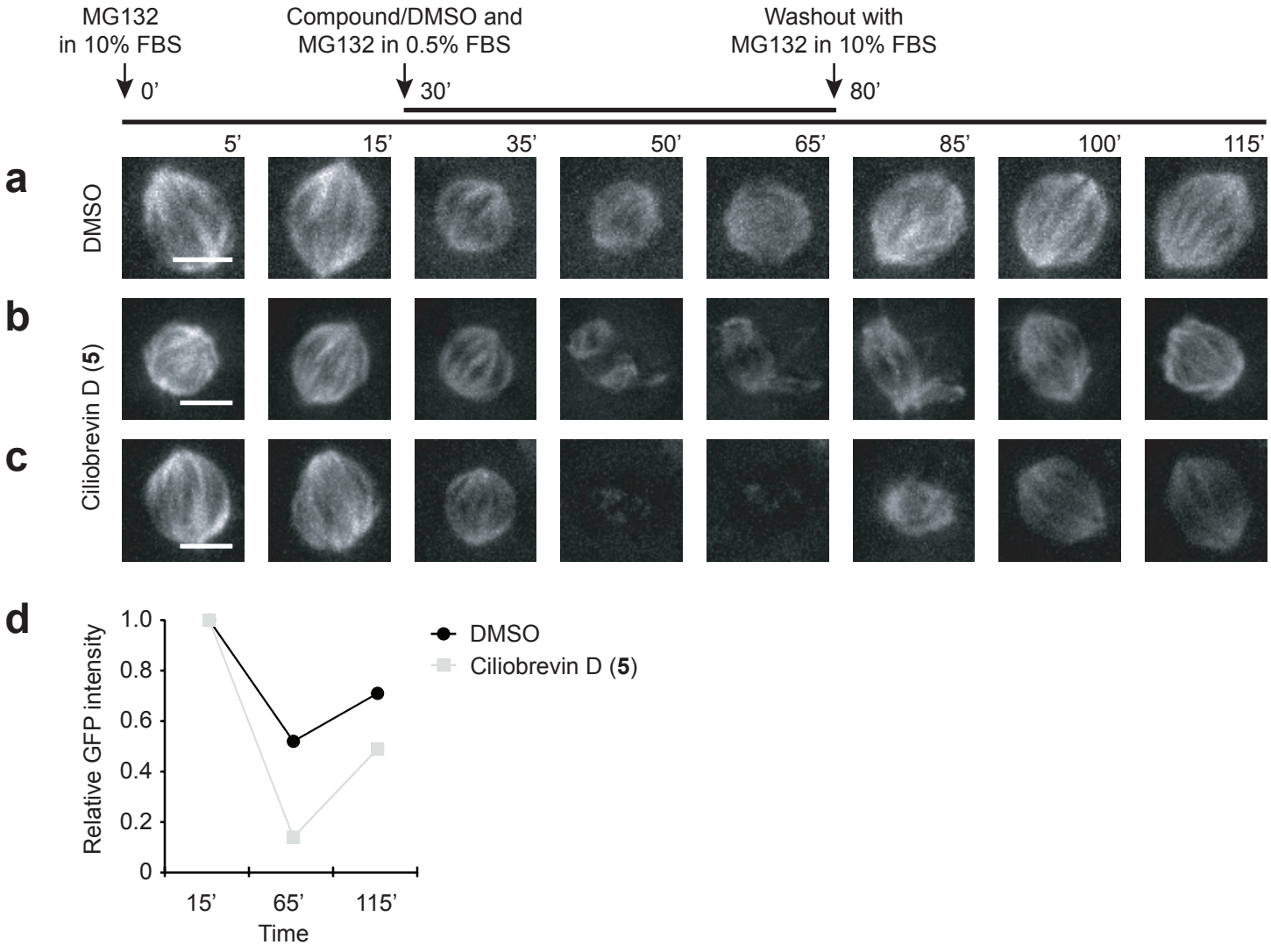
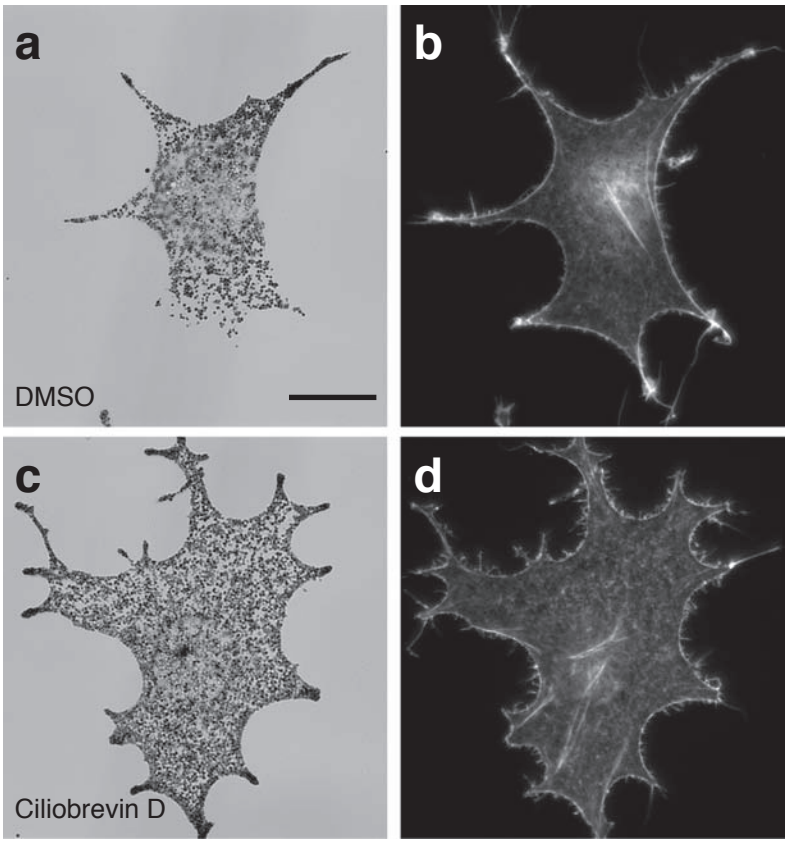
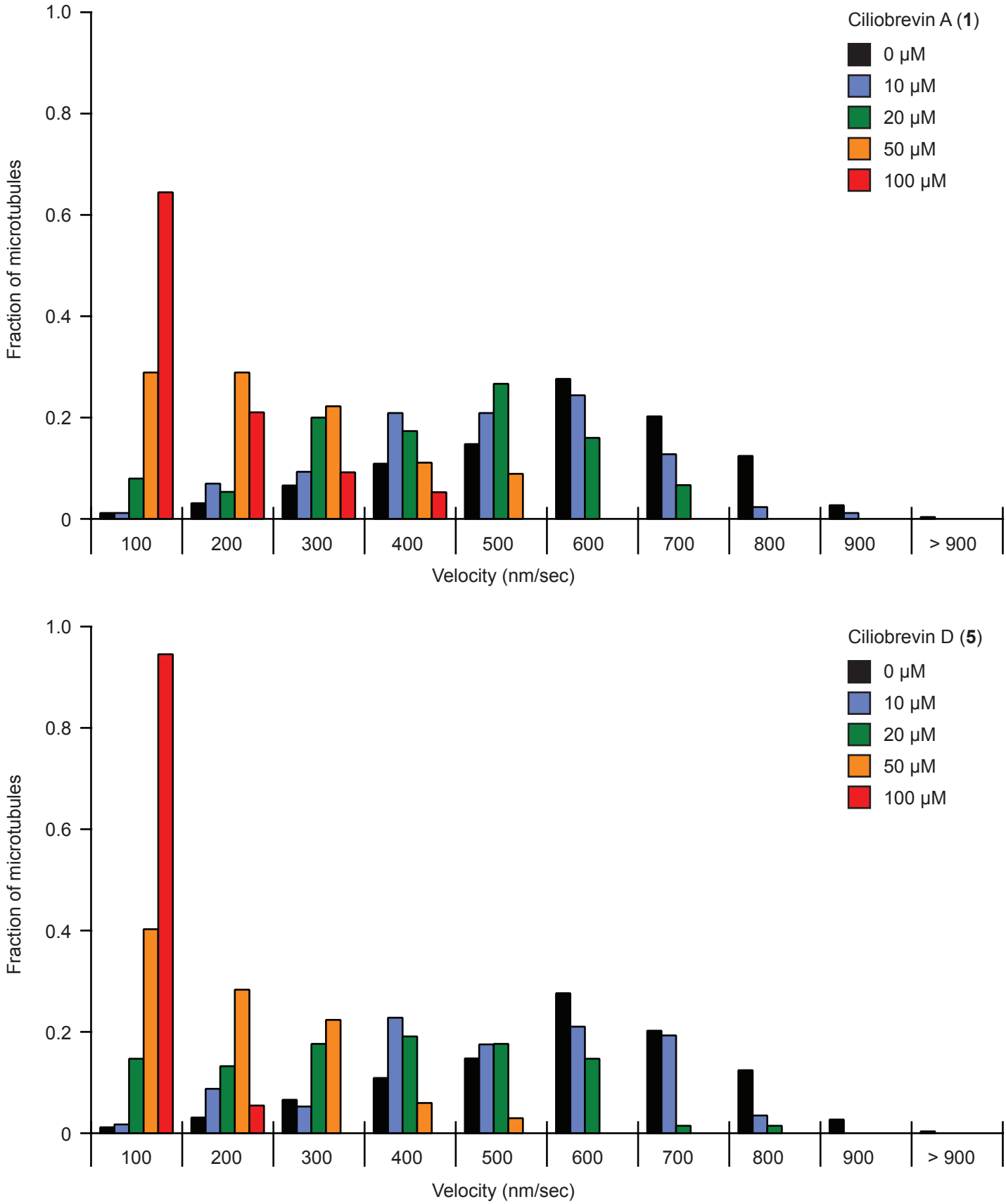


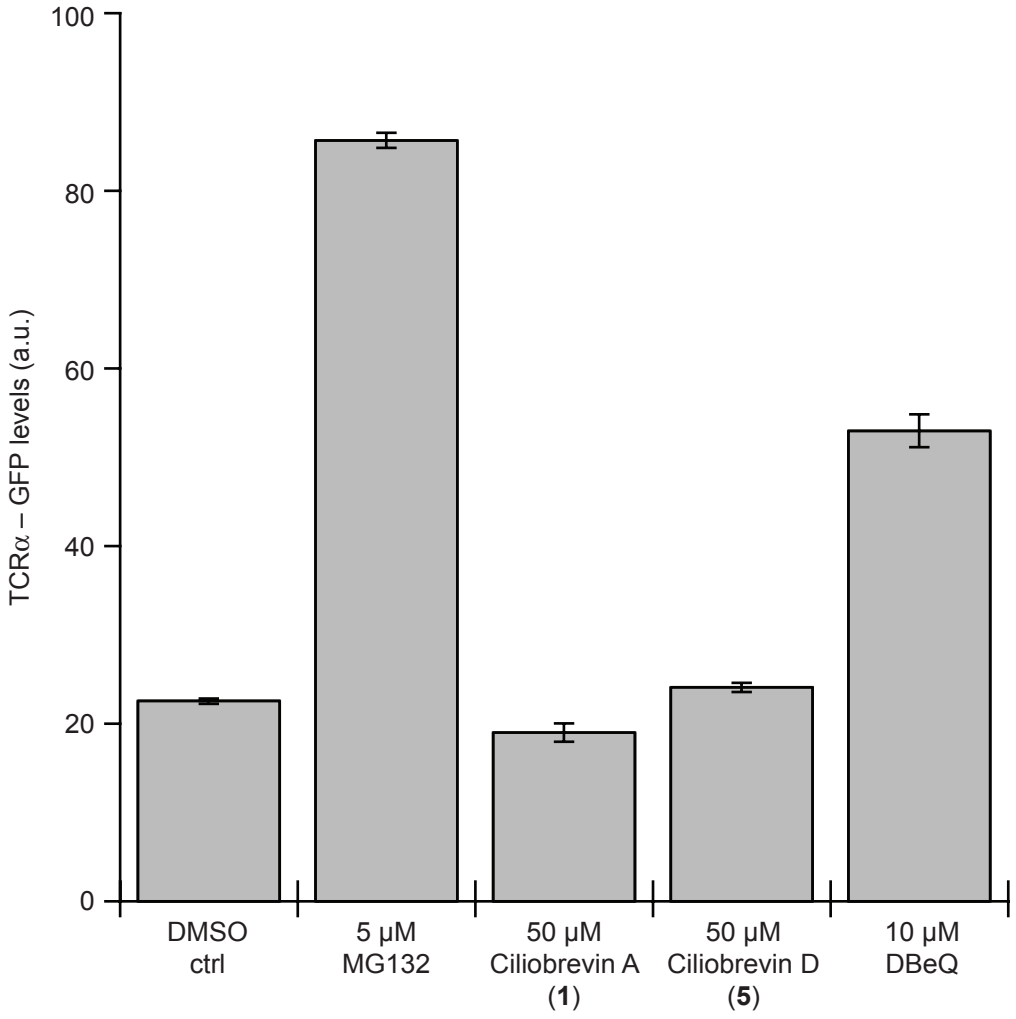
Supplementary Figure 8. Reversible effects of ciliobrevins on spindles of MG132-arrested cells. a-d, NIH-3T3 cells stably expressing GFP-tubulin were arrested in mitosis with 10 μM MG132 and subsequently treated with DMSO (**a**) or 30 μM ciliobrevin D (**5**) (**b-c**). The compounds were later washed out at the indicated time. Times (min) relative to the initial MG132 treatment are shown. Representative examples of ciliobrevin D-treated cells show recovery of the spindle into a bipolar morphology after severe pole disruption (**b**) or severe microtubule depolymerization (**c**). Addition of the inactive analog did not induce either effect. Scale bars: 5 μm. **d**, Quantification of GFP-tubulin intensities of images corresponding to the indicated times, relative to the initial intensity for each condition. Data are the average of 2-3 cells.



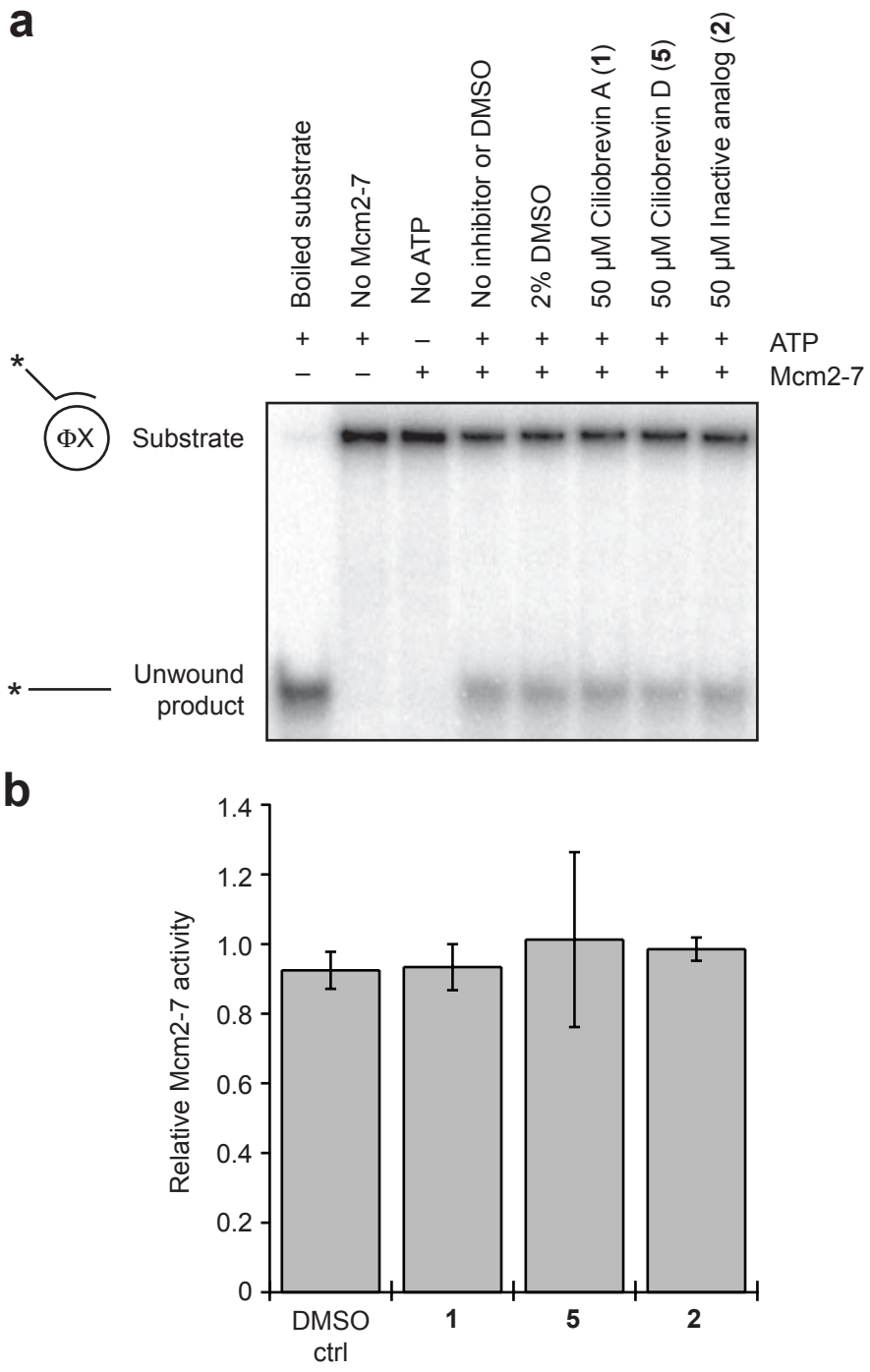
Supplementary Figure 9. Ciliobrevins do not alter the actin cytoskeleton. Rhodamine-conjugated phalloidin staining of melanophores after treatment with DMSO (**a-b**) or 20 μM ciliobrevin D (**5**) (**c-d**). Brightfield (**a** and **c**) and fluorescence (**b** and **d**) images are shown. Scale bar, 10 μm.



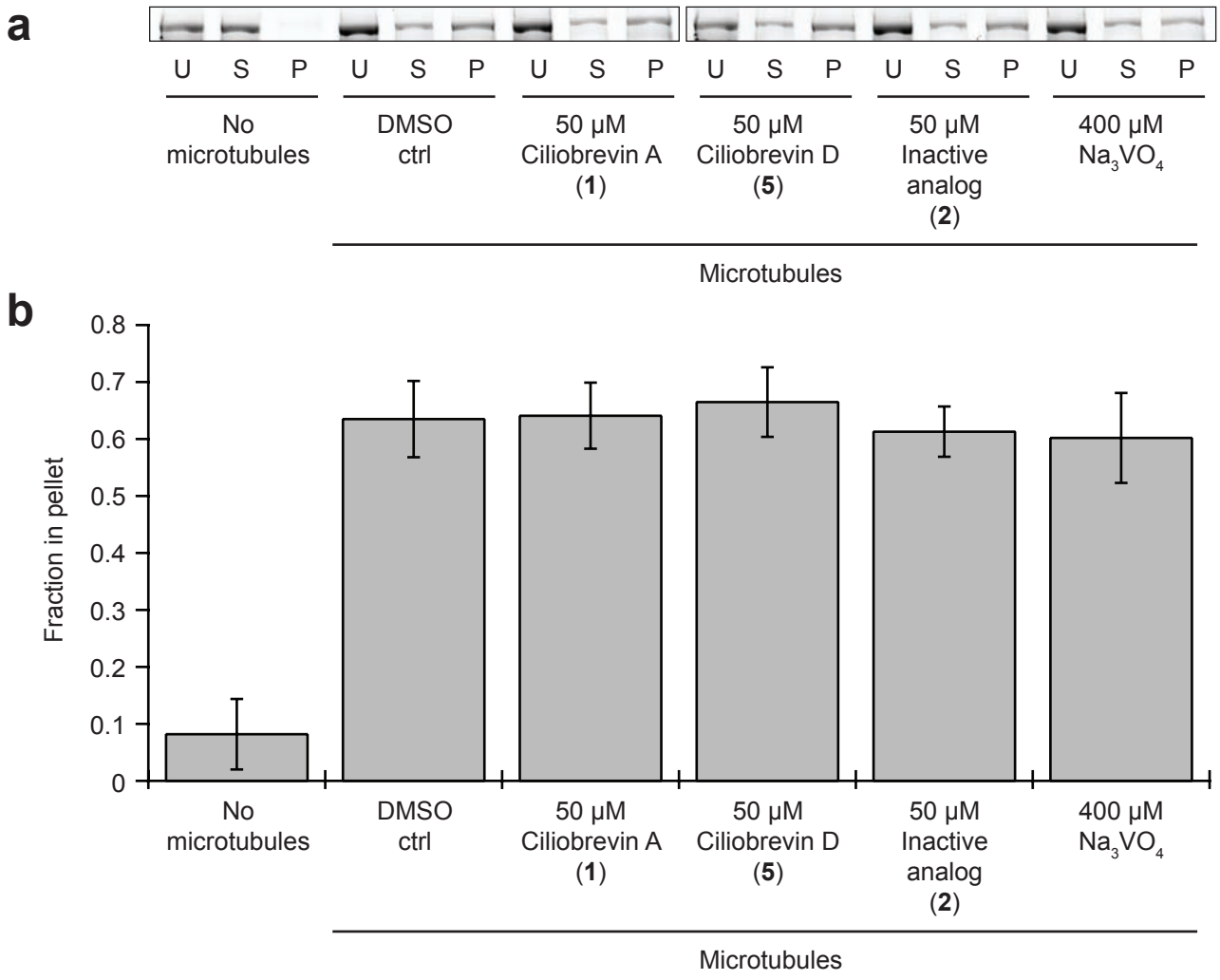
Supplementary Figure 10. Ciliobrevins inhibit dynein-dependent microtubule gliding in a dose-dependent manner. Microtubule velocity distributions associated with different concentrations of ciliobrevin A (**1**) or ciliobrevin D (**5**). At least 45 microtubules were analyzed for each experimental condition.



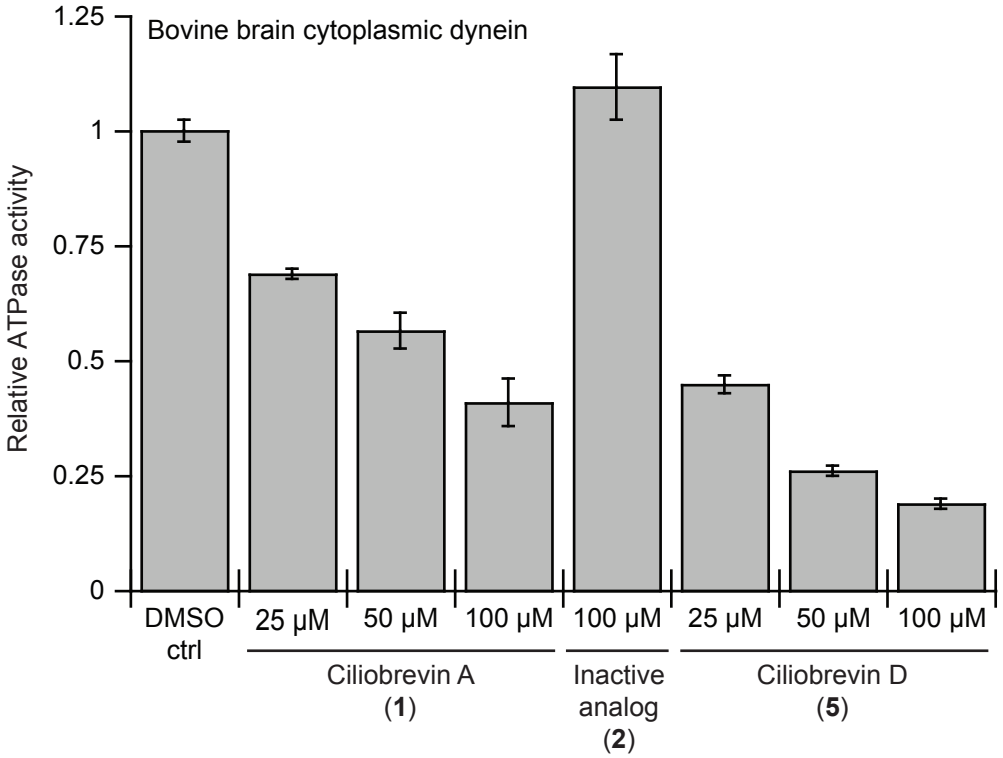
Supplementary Figure 11. Ciliobrevins do not inhibit p97-dependent degradation of endoplasmic reticulum-associated proteins. Effects of DMSO, MG132, ciliobrevin A and D (**1** and **5**), and the p97 inhibitor DBeQ on cellular levels of T-cell receptor α-chain fused to GFP, a reporter of p97-dependent endoplasmic reticulum-associated protein degradation. Data are the average of three independent experiments ± s.d.



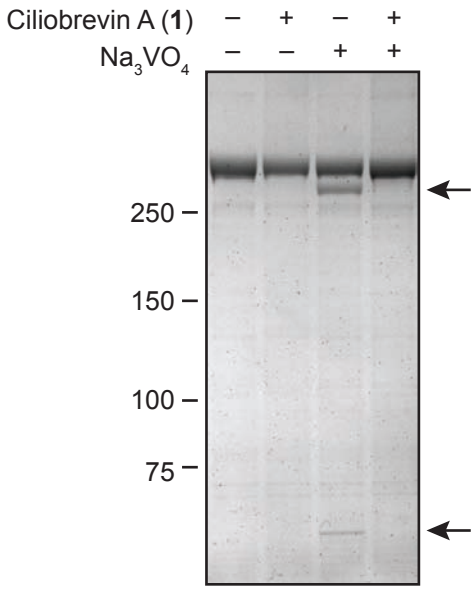
Supplementary Figure 12. Ciliobrevins do not inhibit DNA unwinding by the Mcm2-7 helicase. Effects of DMSO, ciliobrevin A and D (**1** and **5**), and an inactive analog (**2**) on the Mcm2-7-mediated unwinding of a ^{32}P-labeled oligonucleotides annealed to θX174 circular virion single-stranded DNA. **a**, Annealed substrate and unwound products for each reaction condition, resolved by polyacrylamide gel electrophoresis and visualized by phosphoimaging. A representative gel is shown. **b**, Quantification of DNA unwinding for each compound treatment condition. Data are normalized to the inhibitor- and DMSO-free control and are the average of two independent experiments ± s.e.m.



Supplementary Figure 13. Ciliobrevins do not disrupt cytoplasmic dynein-microtubule binding. Effects of DMSO, ciliobrevins A and D (**1** and **5**), an inactive analog (**2**), and Na_3VO_4 on the binding of microtubules to ADP-complexed bovine brain cytoplasmic dynein, as determined by their co-sedimentation upon centrifugation. **a**, Dynein heavy chain levels in uncentrifuged mixtures (U), supernatant (S), and pellet (P) for each experimental condition. Each mixture was electrophoretically resolved on a polyacrylamide gel and stained with colloidal Commassie Blue G-250. A representative gel is shown. **b**, Quantification of dynein heavy chain levels for each condition. Data are the average of four independent experiments ± s.d.



Supplementary Figure 14. Ciliobrevins inhibit the ATPase activity of bovine brain cytoplasmic dynein in a dose-dependent manner. Effects of ciliobrevins A and D (**1** and **5**) and an inactive analog (**2**) on the ATPase activities of cytoplasmic dynein purified from bovine brain. Average ATPase activities for three independent experiments ± s.d. are shown.



Supplementary Figure 15. Ciliobrevins inhibit ultraviolet light-dependent cleavage of sodium vanadate- and ATP-treated cytoplasmic dynein. Effects of ultraviolet irradiation on recombinant rat dynein motor domain incubated with ATP and various combinations of DMSO, 150 μM ciliobrevin A (**1**), and 100 μM Na_3VO_4. Each reaction mixture was electrophoretically resolved on an polyacrylamide gel and stained with Commassie Brilliant Blue R-250. Cleavage products are indicated by the arrows.

Materials and Methods

Compound syntheses

Benzoyl dihydroquinazolinone analog **9** was purchased from Chembridge; all other structural derivatives were synthesized as described below. All chemicals used for organic synthesis were purchased from Sigma-Aldrich or Acros and used without further purification. Anhydrous conditions were maintained under N_2 using standard Schlenk line techniques and oven-dried glassware. ^{1}H and ^{13}C NMR spectra were taken on Varian Inova 300, 400 and 500 MHz spectrometers in DMSO-d_6, and chemical shifts are reported as parts per million (ppm) downfield of the DMSO solvent peak. High-resolution mass spectrometry (HRMS) data were obtained on a Micromass Q-TOF hybrid quadrupole liquid chromatography-mass spectrometer at the Stanford University Mass Spectrometry Facility.

3-(2,4-dichlorophenyl)-3-oxo-2-(4-oxo-3,4-dihydroquinazolin-2(1*H*)-ylidene)propane-nitrile (HPI-4; ciliobrevin A; 1)

2-cyanoethanethioamide (1.00 g, 10.0 mmol) and bromoethane (821 μL, 11.0 mmol) were added to an ethanolic solution of sodium ethoxide (11.5 mmol, 5.3 mL). The resulting mixture was stirred for 6 hours, 2-aminobenzoic acid (1.50 g, 10.9 mmol) was added, and the reaction was refluxed overnight with stirring. A solid precipitate formed upon cooling of the reaction mixture, which was recovered by vacuum filtration and washed sequentially with ethanol, water, ethanol, and diethyl ether. The solid was then dried to yield 2-(4-oxo-3,4-dihydroquinazolin-2-yl)acetonitrile (872 mg, 47%). ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 4.17

(s, 2H), 7.53 (t, J = 7.5 Hz, 1H), 7.68 (d, J = 7.9 Hz, 1H), 7.83 (t, J = 7.8 Hz, 1H), 8.10 (dd, J_1 = 7.9 Hz, J_2 = 1.2 Hz, 1H).

To a solution of 2-(4-oxo-1,4-dihydroquinazolin-2-yl)acetonitrile (202 mg, 1.09 mmol) and triethylamine (167 μL, 1.20 mmol) in dioxane (8 mL) was added 2,4-dichlorobenzoyl chloride (152 μL, 1.09 mmol), and the resulting mixture was refluxed overnight with stirring. A solid precipitate formed upon cooling of the reaction mixture, which was recovered by vacuum filtration and washed sequentially with methanol, water, methanol, and dichloromethane. The solid was then dried to yield ciliobrevin A **(1)** (213 mg, 53%). ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 7.46-7.50 (m, 1H), 7.52 (d, J = 8.2 Hz, 1H), 7.56 (dd, J_1 = 8.2, J_2 = 1.9 Hz, 1H), 7.77 (d, J = 2.0 Hz, 1H), 7.83-7.88 (m, 2H), 8.07 (d, J = 8.1 Hz, 1H). ^{13}C NMR (500 MHz, DMSO-d_6) δ ppm 71.74, 117.43, 117.54, 118.71, 125.92, 126.68, 127.66, 129.21, 129.66, 130.49, 134.85, 135.85, 138.15, 139.25, 155.01, 158.3, 188.75. HRMS (m/z): $[M + Na]^+$ calc. for $C_{17}H_9N_3O_2Cl_2Na$, 379.9970; observed, 379.9967.

3-oxo-2-(4-oxo-3,4-dihydroquinazolin-2(1*H*)-ylidene)-3-phenylpropanenitrile (2)

To a solution of 2-(4-oxo-1,4-dihydroquinazolin-2-yl)acetonitrile (42.8 mg, 231 μmol) and triethylamine (38.7 μL, 277 μmol) in dioxane (4 mL) was added benzoyl chloride (32.2 μL, 277 μmol), and the resulting mixture was refluxed overnight with stirring. A solid precipitate formed upon cooling of the reaction mixture, which was recovered by vacuum filtration and washed sequentially with methanol, water, methanol, and dichloromethane. The solid was then dried to yield analog **2** (36.4 mg, 54%). ^{1}H NMR (500 MHz, DMSO-d_6) δ ppm 7.47 (t, J = 7.5Hz, 1H), 7.49-7.53 (m, 2H), 7.54-7.58 (m, 1H), 7.70-7.73 (m, 2H), 7.84 (td, J_1 = 7.7 Hz, J_2 = 1.5 Hz, 1H), 7.89 (br. d, J = 8.1 Hz, 1H), 8.06 (dd, J_1 = 8.1, J_2 = 1.5 Hz, 1H). ^{13}C NMR (500 MHz, DMSO-d_6) δ ppm 69.3, 117.26, 118.57, 118.81, 125.65, 126.65, 127.59, 128.14, 131.22,

135.77, 139.26, 139.33, 156.12, 158.39, 191.18. HRMS (*m*/*z*): $[M + Na]^+$ calc. for $C_{17}H_{11}N_3O_2Na$, 312.0749; observed, 312.0735.

2-(5-chloro-4-oxo-3,4-dihydroquinazolin-2(1*H*)-ylidene)-3-(2,4-dichlorophenyl)-3-oxo-propanenitrile (ciliobrevin B; 3)

2-cyanoethanethioamide (300 mg, 3.00 mmol) and bromoethane (261 µL, 3.50 mmol) were added to an ethanolic solution of sodium ethoxide (3.50 mmol, 2.5 mL). The resulting mixture was stirred for 6 hours, 2-amino-6-chlorobenzoic acid (500 mg, 2.91 mmol) was added, and the reaction was refluxed overnight with stirring. A solid precipitate formed upon cooling of the reaction mixture, which was recovered by vacuum filtration and washed sequentially with ethanol, water, ethanol, and diethyl ether. The solid was then dried to yield 2-(5-chloro-4-oxo-1,4-dihydroquinazolin-2-yl)acetonitrile (148 mg, 22%). ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 4.15 (s, 2H), 7.54 (dd, J = 7.8 Hz, J_2 = 1.1 Hz, 1H), 7.61 (dd, J_1 = 8.2 Hz, J_2 = 1.1 Hz, 1H), 7.74 (t, J = 8.0 Hz, 1H).

To a solution of 2-(5-chloro-4-oxo-1,4-dihydroquinazolin-2-yl)acetonitrile (26.4 mg, 120 µmol) and triethylamine (18.4 µL, 132 µmol) in dioxane (4 mL) was added 2,4-dichlorobenzoyl chloride (18.5 µL, 132 µmol), and the resulting mixture was refluxed overnight with stirring. A solid precipitate formed upon cooling of the reaction mixture, which was recovered by vacuum filtration and washed sequentially with methanol, water, methanol, and dichloromethane. The solid was then dried to yield ciliobrevin B (**3**) (12.8 mg, 27%). ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 7.46 (dd, J_1 = 7.5 Hz, J_2 = 1.4 Hz, 1H), 7.50 (d, J = 8.2 Hz, 1H), 7.55 (dd, J_1 = 8.2 Hz, J_2 = 1.9 Hz, 1H), 7.68-7.79 (m, 3H). ^{13}C NMR (500 MHz, DMSO-d_6) δ ppm 71.69, 114.6, 117.63, 118.45, 127.65, 128.1, 129.2, 129.65, 130.49, 133.35, 134.8, 135.47,

138.2, 154.81, 156.48, 188.71. HRMS (*m*/*z*): $[M + Na]^+$ calc. for $C_{17}H_8N_3O_2Cl_3Na$, 413.9580; observed, 413.9582.

2-(6-chloro-4-oxo-3,4-dihydroquinazolin-2(1*H*)-ylidene)-3-(2,4-dichlorophenyl)-3-oxo-propanenitrile (ciliobrevin C; 4)

2-cyanoethanethioamide (501 mg, 5.00 mmol) and bromoethane (448 µL, 6.00 mmol) were added to an ethanolic solution of sodium ethoxide (6.00 mmol, 3 mL). The resulting mixture was stirred for 6 hours, 2-amino-5-chlorobenzoic acid (858 mg, 5.00 mmol) was added, and the reaction was refluxed overnight with stirring. A solid precipitate formed upon cooling of the reaction mixture, which was recovered by vacuum filtration and washed sequentially with ethanol, water, ethanol, and diethyl ether. The solid was then dried to yield 2-(6-chloro-4-oxo-1,4-dihydroquinazolin-2-yl)acetonitrile (314 mg, 29%). ^{1}H NMR (500 MHz, DMSO-d_6) δ ppm 4.18 (s, 2H), 7.70 (d, J = 8.8 Hz, 1H), 7.85 (dd, J_1 = 8.7 Hz, J_2 = 2.0 Hz, 1H) 8.03 (d, J = 2.4 Hz, 1H).

To a solution of 2-(6-chloro-4-oxo-1,4-dihydroquinazolin-2-yl)acetonitrile (52.0 mg, 237 µmol) and triethylamine (36.3 µL, 260 µmol) in dioxane (4 mL) was added 2,4-dichlorobenzoyl chloride (33.1 µL, 237 µmol) and the resulting mixture was refluxed overnight with stirring. A solid precipitate formed upon cooling of the reaction mixture, which was recovered by vacuum filtration and washed sequentially with methanol, water, methanol, and dichloromethane. The solid was then dried to yield ciliobrevin C (**4**) (6.4 mg, 7%). ^{1}H NMR (500 MHz, DMSO-d_6) δ ppm 7.50 (d, J_1 = 8.3 Hz, 1H), 7.56 (dd, J_1 = 8.3 Hz, J_2 = 1.9 Hz, 1H), 7.77 (d, J = 1.9 Hz, 1H), 7.83 (d, J_1 = 8.8 Hz, 1H), 7.88 (dd, J_1 = 8.9 Hz, J_2 = 2.3 Hz, 1H), 8.00 (d, J_2 = 2.4 Hz, 1H). ^{13}C NMR (500 MHz, DMSO-d_6) δ ppm 72.1, 117.52, 118.98, 121.22,

125.61, 127.67, 129.22, 129.66, 129.99, 130.49, 134.88, 135.64, 38.11, 138.58, 155.05, 157.52, 188.76. HRMS (*m*/*z*): $[M + Na]^+$ calc. for $C_{17}H_8N_3O_2Cl_3Na$, 413.9580; observed, 413.9583.

2-(7-chloro-4-oxo-3,4-dihydroquinazolin-2(1*H*)-ylidene)-3-(2,4-dichlorophenyl)-3-oxo-propanenitrile (ciliobrevin D; 5)

2-cyanoethanethioamide (300 mg, 3.00 mmol) and bromoethane (299 µL, 4.00 mmol) were added to an ethanolic solution of sodium ethoxide (4.00 mmol, 3 mL). The resulting mixture was stirred for 6 hours, 2-amino-4-chlorobenzoic acid (500 mg, 2.91 mmol) was added, and the reaction was refluxed overnight with stirring. A solid precipitate formed upon cooling of the reaction mixture, which was recovered by vacuum filtration and washed sequentially with ethanol, water, ethanol, and diethyl ether. The solid was then dried to yield 2-(7-chloro-4-oxo-1,4-dihydroquinazolin-2-yl)acetonitrile (118 mg, 18%). ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 4.19 (s, 2 H), 7.57 (dd, J_1 = 8.5 Hz, J_1 = 2.1 Hz, 1H), 7.74 (d, J_1 = 2.0 Hz, 1H), 8.09 (d, J = 8.4 Hz, 1H).

To a solution of 2-(7-chloro-4-oxo-1,4-dihydroquinazolin-2-yl)acetonitrile (24.9 mg, 113 µmol) and triethylamine (17.4 µL, 125 µmol) in dioxane (4 mL) was added 2,4-dichlorobenzoyl chloride (15.9 µL, 113 µmol), and the resulting mixture was refluxed overnight with stirring. A solid precipitate formed upon cooling of the reaction mixture, which was recovered by vacuum filtration and washed sequentially with methanol, water, methanol, and dichloromethane. The solid was then dried to yield ciliobrevin D (**5**) (11.4 mg, 26%). ^{1}H NMR (500 MHz, DMSO-d_6) δ ppm 7.47-7.52 (m, 2H), 7.56 (dd, J_1 = 8.2 Hz, J_2 = 1.8 Hz , 1H), 7.77 (d, J_1 = 1.8 Hz, 1H), 7.90 (br s, 1H), 8.04 (d, J = 8.5 Hz, 1H). ^{13}C NMR (500 MHz, DMSO-d_6) δ ppm 72.44, 116.56, 118.79, 118.85, 125.74, 127.65, 128.66, 129.2, 129.65, 130.49, 134.81,

138.21, 140.03, 155.62, 157.98, 188.6. HRMS (*m*/*z*): $[M + Na]^+$ calc. for $C_{17}H_8N_3O_2Cl_3Na$, 413.9580; observed, 413.9583.

3-(2-chlorophenyl)-3-oxo-2-(4-oxo-3,4-dihydroquinazolin-2(1*H*)-ylidene)propanenitrile (6)

To a solution of 2-(4-oxo-1,4-dihydroquinazolin-2-yl)acetonitrile (22.0 mg, 119 μmol) and triethylamine (18.2 μL, 131 μmol) in dioxane (3 mL) was added 2-chlorobenzoyl chloride (20.8 μL, 164 μmol), and the resulting mixture was refluxed overnight with stirring. A solid precipitate formed upon cooling of the reaction mixture, which was recovered by vacuum filtration and washed sequentially with methanol, water, methanol, and dichloromethane. The solid was then dried to yield analog **6** (12.5 mg, 33%). ^{1}H NMR (300 MHz, DMSO-d_6) δ ppm 7.43-7.52 (m, 4H), 7.52-7.57 (m, 1H), 7.81-7.87 (m, 2H), 8.06 (d, *J* = 8.1 Hz, 1H). ^{13}C NMR (500 MHz, DMSO-d_6) δ ppm 71.7, 117.41, 117.62, 118.73, 125.81, 126.68, 127.33, 128.22, 129.18, 129.57, 131.1, 135.81, 139.33, 155.12, 158.36, 189.89. HRMS (*m*/*z*): $[M + Na]^+$ calc. for $C_{17}H_{10}N_3O_2Cl_1Na$, 346.0359; observed, 346.0373.

3-(3-chlorophenyl)-3-oxo-2-(4-oxo-3,4-dihydroquinazolin-2(1*H*)-ylidene)propanenitrile (7)

To a solution of 2-(4-oxo-1,4-dihydroquinazolin-2-yl)acetonitrile (152 mg, 0.820 mmol) and triethylamine (126 μL, 0.904 mmol) in dioxane (10 mL) was added 3-chlorobenzoyl chloride (116.3 μL, 0.904 mmol), and the resulting mixture was refluxed overnight with stirring. A solid precipitate formed upon cooling of the reaction mixture, which was recovered by vacuum filtration and washed sequentially with methanol, water, methanol, and dichloromethane. The solid was then dried to yield analog **7** (172 mg, 65%). ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 7.48 (t, *J* = 7.5 Hz, 1H), 7.55 (t, *J* = 7.8 Hz, 1H), 7.61-7.87 (m, 3H), 7.85 (t, *J* = 7.6 Hz, 1H), 7.90 (d, *J* = 8.2 Hz, 1H), 8.07 (d, *J* = 8.2 Hz, 1H). ^{13}C NMR (500 MHz, DMSO-d_6) δ ppm 69.62,

117.34, 118.62, 118.73, 25.78, 126.23, 26.65, 127.28, 130.22, 130.9, 132.88, 135.81, 141.15, 155.96, 158.36, 189.3. HRMS (*m*/*z*): $[M + Na]^+$ calc. for $C_{17}H_{10}N_3O_2Cl_1Na$, 346.0359; observed, 346.0368.

3-(4-chlorophenyl)-3-oxo-2-(4-oxo-3,4-dihydroquinazolin-2(1*H*)-ylidene)propanenitrile (8)

To a solution of 2-(4-oxo-1,4-dihydroquinazolin-2-yl)acetonitrile (54.2 mg, 293 μmol) and triethylamine (49.0 μL, 351 μmol) in dioxane (7 mL) was added 4-chlorobenzoyl chloride (45.1 μL, 351 μmol), and the resulting mixture was refluxed overnight with stirring. A solid precipitate formed upon cooling of the reaction mixture, which was recovered by vacuum filtration and washed sequentially with methanol, water, methanol, and dichloromethane. The solid was then to yield analog **8** (59.9 mg, 63%). ^{1}H NMR (500 MHz, DMSO-d_6) δ ppm 7.47 (t, J = 7.6 Hz, 1H), 7.57-7.60 (m, 2H), 7.72-7.75 (m, 2H), 7.84 (t, J = 7.8 Hz, 1H), 7.89 (d, J = 8.2 Hz, 1H) 8.06 (d, J = 8.1 Hz, 1H). ^{13}C NMR (500 MHz, DMSO-d_6) δ ppm 69.44, 117.31, 118.68, 118.73, 125.73, 126.65, 128.29, 129.54, 135.79, 135.88, 137.96, 156.02, 158.37, 189.76. HRMS (*m*/*z*): $[M + Na]^+$ calc. for $C_{17}H_{10}N_3O_2Cl_1Na$, 346.0359; observed, 346.0359.

Shh-LIGHT2 assay for Hh pathway activity

Shh-N-conditioned medium was prepared as previously described[1]. Shh-LIGHT2 cells[2], an NIH-3T3-based cell line containing a stably integrated Gli-responsive firefly luciferase reporter and a constitutive thymidine kinase promoter-driven *Renilla* luciferase expression construct (pRLTK, Promega), were cultured in DMEM containing 10% (v/v) calf serum, 100 U/mL penicillin, and 0.1 mg/mL streptomycin. The cells were seeded into 96-well plates at a density of 30,000 cells/well, and the following day they were treated with individual

dihydroquinazolinones or DMSO in DMEM containing 0.5% (v/v) calf serum, 10% (v/v) Shh-conditioned medium, and the antibiotics described above. After being cultured for another 24 hours, the cells were lysed and their firefly and *Renilla* luciferase activities were determined using a dual luciferase reporter kit (Promega) and a microplate luminometer (Veritas). Average values representing the firefly luciferase/*Renilla* luciferase ratio from triplicate samples were used to construct dose-response profiles for each compound. Dose-response data from three separate experiments were independently curve-fitted with a variable slope, sigmoidal dose-response algorithm using Prism software, and the resulting IC_{50} values were used to generate an average IC_{50} for each compound.

Quantitative assessment of ciliogenesis

Shh-EGFP cells[3], an NIH-3T3-based cell line containing a stably integrated Gli-responsive enhanced green fluorescent protein reporter, were maintained in DMEM containing 10% (v/v) calf serum, 100 U/mL penicillin, and 0.1 mg/mL streptomycin. The cells were seeded into 24-well plates containing poly-D-lysine-coated 12-mm glass coverslips at a density of 30,000 cells/well and cultured for 36 hours. The cells were transferred into DMEM containing 0.5% (v/v) calf serum, the antibiotics described above, and either individual dihydroquinazolinones at a 30 μM concentration or an equivalent amount of DMSO vehicle (0.3%, v/v). After being cultured for another 24 hours, the cells were fixed in PBS containing 4% (w/v) paraformaldehyde for 10 min at room temperature, washed 3 x 5 min with PBS, permeabilized with PBS containing 0.3% (v/v) Triton X-100 for 5 min, and blocked overnight at 4 °C with PBS containing 5% (v/v) normal goat serum, 0.1% (v/v) Triton X-100, and 0.05% (w/v) sodium azide. The coverslips were then incubated for 90 min at room temperature with

blocking buffer containing rabbit polyclonal anti-Arl13b antibody (1:3000 dilution), washed 4 x 5 min with PBS containing 0.1% Triton (v/v) X-100, and incubated for 60 min with blocking buffer containing Alexa Fluor 488-conjugated goat polyclonal anti-rabbit IgG antibody (1:300 dilution; Invitrogen, A-11034). Following this secondary antibody incubation, nuclei were stained by incubating the cells 2 x 10 min with PBS containing 0.1% (v/v) Triton X-100 and 0.5 μg/mL 4',6-diamidino-2-phenylindole (DAPI) and washing them 2 x 5 min with PBS. The coverslips were subsequently mounted onto slides using Prolong Gold Antifade Reagent (Invitrogen) and imaged using an HC Plan Apochromat CS 20x/0.70 NA oil immersion objective on a Leica DMI6000B compound microscope equipped with a Photometric CoolSNAP HQ CCD camera and Metamorph software (Molecular Devices). Images of Arl13b staining in DMSO-treated cells were manually examined to establish a minimum threshold value for cilia staining intensity. ImageJ software was then used to define objects consisting of two or more adjacent pixels with a signal intensity equal to or greater than this threshold value, and the total object area within an individual image was used to estimate the total pixel area of primary cilia. Corresponding images of DAPI staining were processed in parallel using CellProfiler software to establish the number of nuclei per image. The average cilia size per cell within an individual image was then determined by dividing the total ciliary pixel area by the number of nuclei. Ten individual images, each containing approximately 150 cells, were used to quantify the average cilia size for each experimental condition.

Quantitative assessment of Hh ligand-dependent Gli3 processing

Shh-EGFP cells were seeded into 12-well plate at a density of 120,000 cells/well in DMEM containing 10% (v/v) calf serum, 100 U/mL penicillin, and 0.1 mg/mL streptomycin.

After being cultured for 24 hours, the cells were transferred into DMEM containing 0.5% (v/v) calf serum, 10% (v/v) Shh-conditioned medium, the antibiotics described above, and either the individual dihydroquinazolinones at a 30 μM concentration or an equivalent amount of DMSO vehicle (0.3%, v/v). The cells were cultured for an additional 16 hours, washed with PBS, and then lysed by incubation on ice with SDS-PAGE loading buffer composed of 8% (v/v) glycerol, 20 mM Tris-HCl, pH 6.8, 2% (w/v) SDS, 100 mM DTT, 1 mM PMSF, 20 mM NaF, 2 mM Na_3VO_4, and an EDTA-free protease inhibitor cocktail (Roche). The lysed samples were heated to 100 °C for 7 min, loaded onto 3-8% Criterion XT Tris-Acetate polyacrylamide gels (Bio-Rad), electrophoresed in XT Tricine buffer (Bio-Rad), and transferred onto PVDF membranes. The membranes were dehydrated with methanol and probed overnight at 4 °C with goat polyclonal anti-Gli3 antibody (0.4 μg/mL; R & D Systems, AF3690) in PBS containing 4% (w/v) non-fat dry milk and 0.01% (v/v) Tween-20 (immunoblot blocking buffer). The blots were then washed 4 x 1 min in PBS and incubated with horseradish peroxidase-conjugated bovine polyclonal anti-goat IgG antibody (0.04 μg/mL; Jackson ImmunoResearch, 805-035-180) in immunoblot blocking buffer for 1 hour at room temperature. The membranes were next washed 4 x 1 min in PBS and visualized using SuperSignal West Dura Extended Duration substrate (Thermo Scientific) and a ChemiDoc XRS imaging system (Bio-Rad). Band intensities of Gli3FL and Gli3R were quantified using Quantity One software (Bio-Rad), and five independent experiments were used to determine the average Gli3FL/Gli3R ratio for each compound. The representative immunoblot image shown in Fig. 1C was acquired using BioMax MR film (Kodak) and digital scanning.

Quantitative assessment of Hh ligand-dependent Gli2 trafficking

Shh-EGFP cells were seeded into 24-well plates containing poly-D-lysine-coated 12-mm glass coverslips at a density of 65,000 cells/well and cultured for 24 hours in DMEM containing 10% (v/v) calf serum, 100 U/mL penicillin, and 0.1 mg/mL streptomycin. The cells were then transferred into DMEM containing 0.5% (v/v) calf serum and the antibiotics described above for 16 hours to promote primary cilia formation. The cells were next transferred into DMEM containing 0.5% (v/v) calf serum, antibiotics, and either 30 μM of the indicated benzoyl dihydroquinazolinones or an equivalent amount of DMSO vehicle (0.3%, v/v). Each compound or vehicle treatment was conducted in the presence or absence of 10% (v/v) Shh-N-conditioned medium, and the cells were cultured under these conditions for 4 hours. The cells were subsequently fixed in PBS containing 4% (w/v) paraformaldehyde for 10 min at room temperature, washed 3 x 5 min in PBS containing 50 mM glycine, permeabilized in PBS containing 0.3% (v/v) Triton X-100 for 5 min, and then blocked overnight at 4 °C in PBS containing 5% (v/v) normal donkey serum, 0.1% (v/v) Triton X-100, and 0.05% (w/v) sodium azide. After blocking was complete, the coverslips were incubated in blocking buffer containing rabbit polyclonal anti-Arl13b antibody (1:3000 dilution), goat polyclonal anti-Gli2 antibody (1:50 dilution; R & D Systems, AF3635), and mouse monoclonal anti-γ-tubulin (1:200 dilution; Sigma-Aldrich, T5326) for 90 min at room temperature. The cells were then washed 4 x 5 min with PBS containing 0.1% (v/v) Triton X-100 and incubated in blocking buffer containing DyLight 488-conjugated donkey polyclonal anti-goat IgG antibody (3 μg/mL; Jackson ImmunoResearch, 705-485-147), DyLight 594-conjugated donkey polyclonal anti-mouse IgG antibody (3 μg/mL; Jackson ImmunoResearch, 715-515-151), and DyLight 649-conjugated donkey polyclonal anti-mouse IgG antibody (3 μg/mL; Jackson ImmunoResearch, 711-495-152)

for 60 min. Following this secondary antibody incubation, nuclei were stained by incubating the cells 2 x 10 min with PBS containing 0.1% (v/v) Triton X-100 and 0.5 μg/mL DAPI and washing them 2 x 5 min with PBS. The coverslips were subsequently mounted onto slides using Prolong Gold Antifade Reagent (Invitrogen) and imaged using a Plan Apochromat 63x/1.4 NA oil immersion objective on a Zeiss AxioImager Z1 upright microscope equipped with an LSM700 laser scanning confocal head. Ciliary Gli2 levels were quantified by determining total pixel intensity within a 0.69 μm-diameter circle at the distal tip of the axoneme and subtracting background fluorescence in an adjacent region of equivalent size. At least 25 cilia were analyzed to determine the average ciliary Gli2 level for each experimental condition.

Quantitative assessment of IFT88 trafficking

Shh-EGFP cells were seeded into 24-well plates containing poly-D-lysine-coated 12-mm glass coverslips at a density of 30,000 cells/well and cultured for 24 hours in DMEM containing 10% (v/v) calf serum, 100 U/mL penicillin, and 0.1 mg/mL streptomycin. The cells were then transferred into DMEM containing 0.5% calf serum and the antibiotics described above for 16 hours to promote primary cilia formation. The cells were next transferred into DMEM containing 0.5% (v/v) calf serum, antibiotics, and either 50 μM of the indicated benzoyl dihydroquinazolinones or an equivalent amount of DMSO vehicle (0.25%, v/v) for 1 hour. The cells were subsequently fixed in PBS containing 4% (w/v) paraformaldehyde for 10 min at room temperature, washed 3 x 5 min in PBS containing 50 mM glycine, permeabilized in PBS containing 0.3% (v/v) Triton X-100 for 5 min, and then blocked overnight at 4 °C in PBS containing 5% (v/v) normal goat serum, 0.1% (v/v) Triton X-100, and 0.05% (w/v) sodium azide. After blocking was complete, the coverslips were incubated in blocking buffer containing

mouse monoclonal anti-acetylated-tubulin (1:4000 dilution; Sigma-Aldrich, T6793), rabbit polyclonal anti-IFT88 antibody (1:70 dilution; ProteinTech Group, 13967-1-AP), and mouse monoclonal anti-γ-tubulin (1:200 dilution; Sigma-Aldrich, T5326) for 90 min at room temperature. The cells were then washed 4 x 5 min with PBS containing 0.1% (v/v) Triton X-100 and incubated in blocking buffer containing Alexa Fluor 488-conjugated goat polyclonal anti-rabbit IgG antibody (1:300 dilution; Invitrogen, A-11034) and Alexa Fluor 647-conjugated goat polyclonal anti-mouse IgG antibody (1:300 dilution; Invitrogen, A-21235). Following this secondary antibody incubation, nuclei were stained by incubating the cells 2 x 10 min with PBS containing 0.1% (v/v) Triton X-100 and 0.5 μg/mL DAPI and washing them 2 x 5 min with PBS. The coverlsips were subsequently mounted onto slides using Prolong Gold Antifade Reagent (Invitrogen) and imaged using a Plan Apochromat 63x/1.4 NA oil immersion objective on a Zeiss AxioImager Z1 upright microscope equipped with an LSM700 laser scanning confocal head. Ciliary IFT levels were quantified essentially as described for Gli2 though no background was subtracted. At least 25 cilia were analyzed to determine the average ciliary IFT88 level for each experimental condition.

Imaging of mitotic spindle assembly

Shh-EGFP cells were cultured until they achieved approximately 70% confluency and then split 1:5 into 24-well plates containing poly-D-lysine-coated 12-mm glass coverslips in DMEM containing 10% calf serum, 100 U/mL penicillin, and 0.1 mg/mL streptomycin. After culturing under these conditions overnight, the cells were transferred into growth medium containing 15 μM MG132 for 90 min, followed by a 60-min incubation with DMEM containing 0.5% calf serum, 15 μM MG132, and 50 μM of the indicated benzoyl dihydroquinazolinones or

an equivalent amount of the DMSO vehicle (0.25%, v/v). The cells were fixed by incubation on ice with methanol chilled to -20 °C, washed 3 x 5 min in PBS, and then blocked for 1 hour at room temperature with PBS containing 5% normal goat serum, 0.1% Triton X-100, and 0.05 sodium azide.

After blocking was complete, the coverslips were incubated in blocking buffer containing mouse monoclonal anti-α-tubulin antibody (1:2000 dilution; Sigma-Aldrich, T6199) and rabbit polyclonal anti-γ-tubulin antibody (1:500 dilution; Sigma-Aldrich, T3559) for 90 min at room temperature. The cells were then washed 4 x 5 min with PBS containing 0.1% Triton X-100 and incubated in blocking buffer containing Alexa Fluor 488-conjugated goat polyclonal anti-rabbit IgG antibody (1:300 dilution; Invitrogen, A-11034) and Alexa Fluor 594-conjugated goat polyclonal anti-mouse IgG antibody (1:300 dilution; Invitrogen, A-11032) for 60 min. Following this secondary antibody incubation, nuclei were stained by incubating the cells 2 x 10 min with PBS containing 0.1% Triton X-100 and 0.5 μg/mL DAPI and washing them 2 x 5 min with PBS. The coverslips were subsequently mounted onto slides using Prolong Gold Antifade Reagent (Invitrogen) and imaged using a Plan Apochromat 63x/1.4 NA oil immersion objective on a Zeiss AxioImager Z1 upright microscope equipped with an LSM700 laser scanning confocal head.

Spindle morphology and cold-treatment experiments in untransfected NIH-3T3 and HeLa cells were conducted as previously described[4]. NIH-3T3 and HeLa cells were cultured in DMEM supplemented with 10% (v/v) calf serum, 100 U/mL penicillin, and 0.1 mg/mL streptomycin. For spindle morphology analysis experiments, the cells were incubated in medium containing 10 μM MG132 for 90 min. The medium was then exchanged with DMEM containing 0.5% (v/v) calf serum, 10 μM MG132, and either 50 μM of the indicated benzoyl

dihydroquinazolinones or an equivalent amount of the DMSO vehicle, and the cells were cultured for an additional 60 min at 37 °C. For cold-treatment experiments, the cells were incubated on ice, and the medium was exchanged with ice-cold DMEM containing 0.5% (v/v) calf serum, 10 μM MG132, and either 50 μM of the indicated compounds or an equivalent amount of the DMSO vehicle. The cells were fixed with 4% (w/v) paraformaldehyde at room temperature, or on ice in the case of cold-treatment experiments, for 10 min. The fixed cells were subsequently washed with TBS-Tx buffer (TBS with 0.1% (v/v) Triton-X) twice and blocked with Abdil buffer (TBS-Tx containing 2% (w/v) bovine serum albumin and 0.02% (w/v) sodium azide) for 10 min at room temperature. Antibodies for immunofluorescence were diluted in Abdil buffer. Primary antibodies used were: mouse polyclonal anti-γ-tubulin (1:500 dilution, Sigma-Aldrich, T6557), FITC-conjugated mouse monoclonal anti-α-tubulin (1:6000 dilution, Sigma-Aldrich, F2168), human CREST anti-serum (1:20000 dilution, a kind gift from W. Brinkley). Secondary antibodies used were: donkey DyLight 649- or 549-conjugated antibodies (1:400 dilution, Jackson Immunoresearch, 709-495-149, 715-505-150). DNA was stained with Hoechst dye (Sigma-Aldrich). Images were acquired as 0.2-μm Z-stacks on a DeltaVision Image Restoration Microscope (Applied Precision Instruments) using a 100x objective and processed by iterative constrained de-convolution using SoftWorX software. Images shown are maximal intensity projections and were cropped and processed using ImageJ.

For live-imaging studies, NIH-3T3 cells stably expressing GFP-tubulin were established by retroviral infection of a pMSCV plasmid made with Gateway technology using standard protocols and puromycin selection. Wash-in experiments of mitotic cells were conducted by adding L-15 medium containing 0.5% fetal bovine serum and either 50 μM ciliobrevin D or an equivalent amount of DMSO. A single differential interference contrast (DIC) image and a Z-

stack (0.5 μm step size) of GFP fluorescence was taken at the indicated times. In washout experiments, the compound or solvent vehicle was removed by exchanging the medium twice with L-15 containing 10% FBS. To study the effects of ciliobrevins on MG132-arrested cells, the GFP-tubulin-expressing cells were incubated in L-15 medium containing 10% fetal bovine serum and 10 μM MG132. Thirty minutes later, the medium was exchanged to L-15 with 0.5% fetal bovine serum, 10 μM MG132, and either 30 uM of the indicated compounds or an equivalent amount of DMSO. Fifty minutes after the wash-in, the compounds were washed out by exchanging the medium twice with L-15 containing 10% fetal bovine serum and 10 μM MG132. The cells were imaged with a Nikon TE2000 confocal microscope equipped using a Plan Apochromat 100X/1.4 NA oil-immersion objective and a PerkinElmer Wallac UltraView confocal head, and an argon ion laser (Solamere). Quantification was carried out with MetaMorph software as described below for individual kinetochores, except that maximal projection images were used and the quantified area encompassed whole spindles.

To quantify interphase microtubule intensities, the GFP-tubulin-expressing NIH-3T3 cells were incubated with L-15 medium containing 0.5% fetal bovine serum and either 30 μM ciliobrevin D or an equivalent amount of DMSO for 15 min. Interphase cells were identified by DIC microscopy, and an 8- to 12-plane Z-stack (0.2 μm step size) encompassing the network of cytoplasmic microtubules was acquired for the GFP channel. For ciliobrevin D experiments, a mitotic cell was imaged before the interphase cells, to ensure microtubule depolymerization had occurred. Ciliobrevin D or DMSO was washed out by exchanging the medium twice with L-15 containing 10% fetal bovine serum 35-50 min after the initial addition of compound or solvent vehicle. The cells were then re-visited and imaged as before, approximately 15 min after the wash-out (at which time the microtubule density of the imaged mitotic cells had recovered). To

quantify microtubule intensity in individual cells before and after ciliobrevin/DMSO wash-out, planes of the Z-stack that showed maximal microtubule intensity were summed, and GFP intensity was measured for a region of interest in the cell cytoplasmic and in a nearby background area of the same size. The background-corrected intensity was calculated as (GFP-tubulin fluorescence – background fluorescence)/background fluorescence, and the corresponding ratio of post- and pre-wash-out intensities was calculated for each cell.

Imaging of kinetochore components

To examine the localization of p150-Glued, ZW10, and CENP-E, NIH-3T3 cells were incubated for 45 min with DMEM containing 0.5% fetal bovine serum and either 50 μM ciliobrevin D, 50 μM analog **2**, or an equivalent amount of DMSO. The cells were then incubated in medium that also contained 1 μg/mL nocodazole (and the respective compounds/DMSO) for another 45 min. The cells were fixed with 4% (w/v) paraformaldehyde at room temperature for 10 min and then subsequently washed twice with TBS-Tx buffer and blocked with Abdil buffer for 15 min. Antibodies for immunofluoresence were diluted in Abdil buffer, and cells were incubated with primary antibodies overnight at 4 °C; secondary antibodies were incubated for one hour at room temperature, and DNA was stained with Hoechst dye (Sigma-Aldrich). Primary antibodies used were: mouse monoclonal anti-p150-Glued (1:500 dilution, BD Biosciences, 610473), rabbit polyclonal anti-CENP-E (1:1000 dilution; a kind gift from Dr. T. Yen), rabbit polyclonal anti-ZW10 (1:500 dilution, Abcam, 21582); human CREST anti-serum (1:20000 dilution).

p150-Glued localization in cells with intact microtubules was also examined by culturing NIH-3T3 cells in DMEM containing 10% fetal bovine serum and 10 μM MG132 for 90 min. The

medium was then exchanged with DMEM containing 0.5% (v/v) fetal bovine serum, 10 μM MG132, and 50 μM ciliobrevin D or an equivalent amount of DMSO. The cells were then fixed and processed for immunostaining as before, using the following primary antibodies: FITC-conjugated mouse monoclonal anti-α-tubulin (1:6000 dilution, Sigma-Aldrich, F2168), human CREST anti-serum (1:20000 dilution), and mouse monoclonal anti-p150-Glued (1:500 dilution, BD Biosciences, 610473). The anti-p150-Glued and CREST antibodies were incubated overnight at 4 °C; all other antibodies were incubated for 1 hour at room temperature.

Images were acquired using 0.2-μm Z-stacks on a DeltaVision Image Restoration Microscope (Applied Precision Instruments) using a 100X objective and SoftWorX software. Maximal intensity projections are shown as final micrographs, with equal scaling for each antibody. Images were cropped and processed using ImageJ. Individual kinetochores were quantified as described[5]. Briefly, using MetaMorph software, integrated fluorescence intensity was measure for a region of interest drawn manually to cover the desired kinetochore at the appropriate plane. This "inner" region was then dilated by four pixels, and the fluorescence intensity for this "outer" region was measured. Background-corrected fluorescence intensity for the kinetochore was then calculated by subtracting the fluorescence intensity in the outer region from the inner region, after scaling for the difference in area. This value was then divided by the size of the inner region to obtain the corrected fluorescence intensity per unit area.

Melanosome aggregation assay

Immortalized *Xenopus* melanophores were cultured in 0.7X L15 medium supplemented with 10% (v/v) fetal bovine serum, 300 mg/L L-glutamine, and 5 mg/L insulin. The cells were cultured on poly-L-lysine-coated coverslips or in 12-well plates for 12 to 24 hours prior to the

melanosome aggregation assay. On the day of the experiment, the melanophores were cultured in serum-free medium for 30 min, stimulated with 100 nM melanocyte-stimulating hormone (MSH) to uniformly disperse the melanosomes, and then incubated with medium containing the indicated benzoyl dihydroquinazolinones or an equivalent amount of DMSO vehicle (0.2%, v/v) for 10 min. Melanosome aggregation was then induced by treating the cells with 10 nM melatonin in the presence of the small molecules. The melanophores were either imaged immediately by time-lapse microscopy or fixed after 30 min with 0.7X PBS containing 4% (w/v) paraformaldehyde. Time-lapse sequences were acquired on an inverted Nikon Eclipse U2000 microscope with a CoolSNAP ES CCD camera (Roper Scientific) using a Plan Apochromat 60X/1.45 NA oil-immersion objective. Fixed cells were imaged on the same system using an S Fluor 40X/1.30 NA objective.

To demonstrate the reversibility of the ciliobrevins, the melanophores were cultured in serum-free medium for 30 min, stimulated with 100 nM melanocyte-stimulating hormone (MSH) to uniformly disperse the melanosomes, and then incubated with medium containing individual compounds and 10 nM melatonin for 30 min. The cells were then washed several times with medium containing melatonin alone and imaged.

Peroxisome motility assay

Drosophila S2 cells stably expressing peroxisome-targeted GFP were cultured and maintained in insect Xpress media (Lonza). In preparation for microscopy, the cells were treated with 5 μM cytochalasin D for 1 hour to depolymerize actin and then plated on glass coverslips pre-coated with concanavalin A. The cells were treated with Xpress medium containing either 200 nM ciliobrevin D or an equivalent amount of DMSO (0.1%, v/v) for 5 min, and the GFP-

labeled peroxisomes were then imaged every second for 1 min with an Orca-II ER CCD camera (Hamamatsu Photonics) on an inverted Nikon Eclipse U2000 microscope, using a Plan Apochromat 100X/1.40 NA oil-immersion objective. An AURA light engine LED light source (LumenCor) was used for fluorescence excitation.

Peroxisome movements were quantitatively analyzed using the u-track software developed by Jaqaman and co-workers[6]. A single vector was defined as the distance moved by a single peroxisome in 1 second. Movement toward and away from the cell center were defined as negative and positive vectors, respectively. 443 peroxisomes in 8 control cells and 376 peroxisomes in 10 ciliobrevin-treated cells were analyzed to generate this data set.

Phalloidin staining of *Xenopus* melanophores

Xenopus melanophores were cultured on poly-L-lysine-coated glass coverslips in 0.7X L15 medium. Prior to imaging, the cells were serum-starved for 30 min in 0.7X L15 medium and then treated for 30 min with either 20 μM ciliobrevin D or an equivalent amount of the DMSO vehicle (0.2%, v/v) in 0.7X L15 medium. The cells were fixed in 0.7X PBS containing 4% (w/v) paraformaldehyde for 15 min at room temperature, permeabilized in 0.7X PBS containing 1% (w/v) bovine serum albumin and 0.1% (v/v) Triton X-100 for 10 min at room termpature, and stained with rhodamine-labeled phalloidin (Molecular Probes/Invitrogen, R415). The cells were washed briefly in PBS, mounted, and imaged. Phalloidin-stained melanophores were imaged on an inverted Nikon Eclipse U2000 microscope using a Plan Apochromat TIRF 60X/1.45 NA oil-immersion objective and a CoolSNAP ES CCD camera. A 100-W halogen lamp was used for fluorescence excitation.

Microtubule surface gliding assay

Cytoplasmic dynein was purified from bovine brains as described[7]. K560, a 560-amino acid N-terminal fragment of human conventional kinesin (kinesin-1) with a C-terminal His-tag, was expressed in bacteria and purified as described[8]. Motility assays were performed on a Zeiss Axiovert 200M wide-field microscope equipped with a Zeiss 100x/1.45 NA α-Plan-Fluar objective. Data were captured with an EM-CCD camera (iXon DU-897, Andor Technology) with a 0.3-second exposure time and frame rate of 0.5 $second^{-1}$.

Microtubule gliding assays were performed as described[9], with some modifications. A 6-μL flow chamber was filled with motor protein (100 μg/mL dynein or 50 μg/mL K560) in motor dilution buffer (80 mM PIPES, 1 mM EGTA, 2 mM $MgCl_2$, 2 mM DTT, 50 μM ATP, pH 6.8 with KOH). After a 2-min incubation, excess protein was washed out with 20 μL PEM80 buffer (80 mM PIPES, 1 mM EGTA, 2 mM $MgCl_2$, pH 6.8 with KOH) and the surface was blocked against non-specific microtubule binding by filling the chamber with blocking protein (0.5 mg/mL α-casein for dynein experiments and 1 mg/mL bovine serum albumin for K560 experiments) in motor dilution buffer. After 2 min the chamber was perfused with 18 μL of reaction mix (PEM80, 40 mM KCl, blocking protein [1 mg/mL α-casein for dynein experiments; 1 mg/mL bovine serum albumin for K560 experiments], 2 mM MgATP, 20 μM taxol, 0.1 μM rhodamine-labeled microtubules, oxygen depletion system [4 mM DTT, 2 mM glucose, 40 μg/mL glucose oxidase, 35 μg/mL catalase], 2.5% (v/v) DMSO, and compounds as appropriate). The flow chamber was then sealed with valap. After allowing the microtubules to bind to the surface for 5 min, the gliding microtubules were visualized by time-lapse fluorescence microscopy. Velocities were measured by kymography using Metamorph software

(Molecular Devices), and the velocity for each microtubule was determined from the total distance during the time observed.

For washout experiments, the chamber was left unsealed after the initial reaction mix containing inhibitor was added. Microtubules were allowed to bind to the surface for 5 min, and then a time-lapse movie was acquired. The inhibitor was then washed out of the chamber by flowing in 20 μL of fresh reaction mix (PEM80, 40 mM KCl, 1 mg/mL α-casein, 2 mM MgATP, 20 μM taxol, oxygen depletion system, and 2.5% (v/v) DMSO) without additional microtubules or inhibitor. The chamber was sealed, and additional time-lapse movies were acquired.

p97-dependent endoplasmic reticulum-associated protein degradation assay

HEK-293 cells expressing the T-cell receptor α-chain fused to GFP (TCRα-GFP)[10] were kindly provided by Ron Kopito. The cells were seeded into 24-well plates at a density of 40,000 cells/well and cultured in DMEM containing 10% (v/v) fetal bovine serum, 100 U/mL penicillin, and 0.1 mg/mL streptomycin. After overnight culture, the cells were cultured for an additional 3 hours in DMEM containing 0.5% (v/v) calf serum, 100 U/mL penicillin, 0.1 mg/mL streptomycin, and either 50 μM of the indicated benzoyl dihydroquinazolinones, 10 μM DBeQ (BioVision), 5 μM MG132, or an equivalent amount of DMSO vehicle (0.5%, v/v). The cells were then dissociated with TrypLE Express (Invitrogen), resuspended in ice-cold PBS containing 3% (v/v) fetal bovine serum, and analyzed on a BD FACSCalibur (Ex: 488 nm; Em: 530/30 nm). At least 7,000 cells of typical size as determined by forward and side scatter properties were used to determine the median value of GFP fluorescence for each condition.

Mcm2-7 helicase assay

Yeast Mcm2-7 was purified as described[11], and yeast RPA was obtained by the alternate protocol reported for human RPA[12]. Each helicase assay contained 10 pmol Mcm2-7, 13 pmol RPA, and 40 fmol θX174 circular virion ssDNA (New England Biolabs) primed with a 5'-radiolabeled oligonucleotide in a 20 μL reaction. The oligonucleotide had a 5' T_{40} tail and the sequence 5'-CAAGCAGTAGTAATTCCTGCTTTATCAAG-3' at the 3' end, complementary to a region in θX174; it was radiolabeled at the 5' end using T4 PNK in the presence of [α-^{32}P] ATP prior to annealing to θX174. Reaction buffer consisted of 20 mM Tris-Acetate, pH 7.6, 1.5 mM magnesium acetate, 100 mM potassium glutamate, 5 mM creatine phosphate, 5 mM creatine kinase, 5 mM DTT, 40 μg/mL bovine serum albumin, 0.1 mM EDTA, 0.01% (v/v) Triton X-100, and where appropriate, 1 mM ATP and either 50 μM of the indicated benzoyl dihydroquinazolinones or an equivalent amount of DMSO vehicle (2%, v/v).

Reactions were assembled on ice, initiated by incubation at 37 °C for 20 min, and then quenched by addition of 2.5 μL of 20 mg/mL proteinase K and further incubation at 37 °C for 5 min. Products were resolved on a 10% native polyacrylamide minigel and visualized by direct phosphoimaging. Band intensities were quantified using ImageQuant TL (GE Healthcare), and Mcm2-7-dependent DNA unwinding was determined as the ratio of unwound product to the sum of product and substrate.

Cytoplasmic dynein-microtubule co-sedimentation assay

Prior to preparing reaction mixtures for each experimental condition, stocks of bovine brain cytoplasmic dynein and bovine serum albumin (fraction V, Sigma-Aldrich, repurified on a Superdex 200 size-exclusion column) were centrifuged at 4 °C in a Beckman TLA120.1 rotor for

10 min at 75,000 rpm to pellet insoluble matter. Individual reaction mixtures were then prepared, each containing 70 mM PIPES, pH 6.8, 120 μg/mL dynein, 2.5 μM microtubules (except in the no-microtubule control), 1 mM ADP, 0.5 mg/mL bovine serum albumin, 3% (v/v) DMSO, and the indicated compounds. The reactions were incubated at room temperature for 20 min, and aliquots were collected from each mixture to acquire uncentrifuged samples. The remaining reaction mixtures were centrifuged at 27 °C in a Beckman TLA 120.1 rotor for 10 min at 67,000 rpm. Supernatants were collected after centrifugation, and the pellets were washed with PEM80 buffer containing 2 mM DTT and 40 μM taxol. The pellets were then resuspended in cold depolymerization buffer (PEM80 buffer containing 50 mM KCl and 10 mM $CaCl_2$).

The uncentrifuged reaction (U), supernatant (S), and pellet (P) for each experimental condition was then electrophoretically resolved on a Tris-glycine 4-20% polyacrylamide gel (Invitrogen), which was fixed with an aqueous solution containing 50% (v/v) methanol and 10% (v/v) acetic acid for 30 min, washed 4 x 10 min with water, and then stained with GelCode Blue Stain Reagent (Fisher Scientific) for 1 hour. After destaining 4 x 10 min with water, the gels were scanned with an Odyssey infrared scanner (Li-Cor Biosciences). Dynein heavy chain band intensities were quantified with the Odyssey imaging software. Fraction co-sedimented (F) was determined using the formula $F = I_P/(I_S + I_P)$, where I_P is the band intensity in the pellet sample and IS is the band intensity in the supernatant sample.

Expression and purification of rat cytoplasmic dynein motor domain

P2 baculovirus for the heterologous expression of a rat cytoplasmic dyenin motor domain (380 kDa; amino acids 1286-4644 with a C-terminal hexahistidine tag) was generously provided by Richard Vallee[13]. The virus was amplified by using 1 mL of the P2 baculovirus stock to

infect a 50-mL shaker flask culture of SF9 cells (4 x 10^5 cells/mL) in SF900-II SFM medium (Invitrogen). After 80 hours of culturing at 28 °C, the flask contents were transferred to a 50-mL conical tube and centrifuged at 1,200 x *g* for 10 min to provide P3 virus stock. Protein production was initiated by using 10 mL of P3 virus to infect a 190-mL culture of SF9 cells (3 x 10^6 cells/mL) in SF900-II SFM medium. Approximately 40 hours of additional culturing at 28 °C, the cells were harvested by centrifugation at 1,200 x *g* for 10 min, resuspended in 6 mL of extraction buffer (25 mM HEPES, 50 mM PIPES, 2 mM $MgSO_4$, 0.2 mM EGTA, pH 7.4) and frozen in liquid nitrogen.

To purify the overexpressed rat dynein motor domain, 3 mL of frozen infected cells were thawed in 50 mL of extraction buffer containing a complete EDTA-free protease inhibitor cocktail (Santa Cruz Biotechnology), 1 mM PMSF, and 14.7 mM β-mercaptoethanol. The cells were then lysed by probe sonication on ice for a total of five 10-pulse cycles. The lysates were clarified by centrifugation at 6,000 x *g* in 50-mL conical tubes, followed by ultracentrifugation at 20,000 rpm in a Beckman SW38 rotor. The resulting supernatant was supplemented with imidazole to achieve a final concentration to 20 mM and then added to 5 mL of Ni-NTA beads (Qiagen) equilibrated in extraction buffer supplemented with 15 mM imidazole. Binding of the hexahistidine-tagged dynein motor domain to the beads was allowed to proceed for 2 hours at 4 °C with gentle agitation. The bead/lysate mixture was then passed through at 1.5 cm x 10 cm fritted column (Kimble-Chase) to collect the beads, which were subsequently washed with 40 mL extraction buffer containing 15 mM imidazole. The bound protein was eluted with extraction buffer containing 150 mM imidazole, and protein-containing elution fraction were dialyzed extensively against buffer containing 12.5 mM HEPES, 25 mM PIPES, 2 mM $MgSO_4$,

and 2 mM EGTA, pH 7.4 to remove imidazole. Protein levels were quantified using a bicinchoninic acid assay (Pierce) and stock solutions were snap frozen in liquid nitrogen.

ATPase assays

The ATPase activity of cytoplasmic dynein purified from bovine brain was determined using the kinesin ELIPA Biochem Kit (Cytoskeleton, BK060). Individual 100-μL reactions were prepared in 96-well plates, each containing ELIPA reaction buffer (15 mM PIPES, 300 mM KCl, 5 mM $MgCl_2$, 0.2 mM 2-amino-6-mercapto-7-methylpurine ribonucleoside, 0.075 units of purine nucleoside phosphorylase, pH 7.0) supplemented with 60 μg/mL dynein, 0.1 mg/mL microtubules, 20 μM taxol, and either the indicated benzoyl dihydroquinazolinones or an equivalent amount of DMSO vehicle (1%, v/v). The reactions were initiated by the addition of ATP to a final concentration of 600 μM, and the absorbance at 360 nm was monitored using a Benchmark Plus Bio Spectophotometer (Bio-Rad) at 37 °C. Linear regions of the resulting absorbance versus time plots and a standard curve of phosphate concentration versus absorbance was used to calculate the velocity of individual reactions. Data represent the average velocities of three independent reactions ± s.d.

The ATPase activity of recombinant rat cytoplasmic dynein motor domain was determined using a Malachite Green-based colorimetric assay (Novus Biologicals, 601-0121). Individual 30-μL reactions were prepared in 96-well PCR plates, each containing ATPase reaction buffer (25 mM PIPES, 30 mM KCl, 5 mM $MgCl_2$, 1 mM EGTA, 1 mM DTT, and 0.01% (v/v) Triton X-100, pH 7.0) supplemented with 47.5 μg/mL dynein, 0.25 mg/mL microtubules, 10 μM taxol, and either the indicated benzoyl dihydroquinazolinones or an equivalent amount of DMSO vehicle (2%, v/v). The reactions were initiated by the addition of

ATP to a final concentration of 1 mM and allowed to proceed for 10 min at 37 °C. 15 μL of each reaction was then added to the wells of clear-bottom 96-well plate containing the Malachite Green reagent. The absorbance at 620 nm was determined for each well using a Benchmark Plus Bio Spectophotometer (Bio-Rad) and compared to a standard curve generated by serial dilutions of KH_2PO_4 to determine the amount of phosphate release. Data represent the average velocities of two independent reactions normalized to the DMSO control ± s.d.

To investigate the mechanism by which the ciliobrevins inhibit cytoplasmic dynein function, the ATPase activity of recombinant rat cytoplasmic dynein motor domain associated with various ATP and compound concentrations was determined as described above with the following differences. Individual 50-μL reactions were prepared in a clear-bottom 96-well plate, each containing ATPase reaction buffer supplemented with 16 μg/mL dynein and either the indicated benzoyl dihydroquinazolinones or an equivalent amount of DMSO vehicle (2%, v/v). The reactions were initiated by the addition of ATP to the indicated final concentrations and allowed to proceed for 30 min at room temperature. Malachite Green was then added to each well, and reaction velocities were determined as before.

The ATPase activity of recombinant human kinesin-1 motor domain (amino acids 5-379; Cytoskeleton, KR01) was determined using reaction conditions and methods identical to those for the rat cytoplasmic dynein motor domain except that 3.3 μg/mL kinesin-1 motor domain was used in place of dynein.

The ATPase activity of recombinant human Eg5 motor domain (amino acids 1-368)[14] was determined using reaction conditions and methods similar to those for the rat cytoplasmic dynein motor domain. In these experiments, individual 30-μL reactions were composed of ATPase reaction buffer supplemented with 8 μg/mL Eg5, 0.20 mg/mL microtubules, 10 μM

taxol, and either the indicated benzoyl dihydroquinazolinones or an equivalent amount of DMSO vehicle (2%, v/v).

Ultraviolet light-dependent cleavage of the cytoplasmic dynein motor domain-ADP-vanadate complex

Individual 40-μL reactions were prepared, each containing ATPase reaction buffer supplemented with 200 μM ATP, 120 μg/mL recombinant rat cytoplasmic dynein motor domain, and either 150 μM ciliobrevin A, 100 μM Na_3VO_4, or an equivalent amount of DMSO vehicle (2%, v/v). The reactions were incubated for 30 at room temperature and then exposed to 365-nm light from a hand-held UV lamp (Spectroline, ENF-240C) for 90 min on ice. Each sample was then treated with 8 μL of 6X SDS-PAGE loading buffer, heated to 98 °C for 7 min, and electrophoretically resolved on a 3-8% Criterion XT Tris-Acetate polyacrylamide gel (Bio-Rad) in XT Tricine buffer. The gels were fixed overnight in an aqueous solution of 50% (v/v) methanol and 10% (v/v) acetic acid, stained with Commassie Brilliant Blue R-250, and imaged using a ChemiDoc XRS imaging system (Bio-Rad).

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