Supplementary Materials and Methods:

Gel filtration:

For gel filtration 1,5mg protein was fractionated on a calibrated Ultrogel AcA34 (Pall Life Sciences, Vienna, Austria) or Superose 6 (GE Healthcare, Munich, Germany) filled Tricorn 10/600 column (GE Healthcare) run on a BioLogic Dual system (BioRad) at 4°C in lysis buffer at 0.5ml/min. 1ml fractions were collected, precipitated with TCA and analysed by immunoblotting as described previously (Geley *et al.*, 2001;Wolf *et al.*, 2006).

Antibodies:

The following antibodies were used: α-tubulin (mouse monoclonal (mAb), TAT-1, Julian Gannon (JG), C.R.U.K. Sout Mimms, UK for blotting, and Sigma, #B512 for staining); Cdk1 (mAb, A17, (JG)); cyclin B1 (mAb, V152, JG); human CREST serum; GAPDH (mAb, High Test Ltd, #6C5); dynamitin (mAb, BD Transduction Laboratories, #25); Dynein intermediate chain (mAb, #70.1, Sigma; #74.1, Chemicon); Dynein heavy chain 1 (rabbit polyclonal antibody (rb), HPA003742, Sigma); FLAG-M2 (mAb, F3165, Sigma); MAD2 (rb, #24588, Abcam, Cambridge, UK); Pericentrin (rb, #4448, Abcam); Rod/KNTC1 (mAb, #10H4, Abnova, Taiwan); Spindly (mouse polyclonal and mAb, #A01, #2F4, Abnova); Spindly (rb, #A301-354A, Bethyl Laboratories Inc., Montgomery, TX, USA); ZW10 (rb, #21582, Abcam,); ZWINT1 (rb, #IHC-00095, Bethyl); Secondary antibodies conjugated with Alexa Fluor 488, 546 and 647 were from Molecular Probes (Invitrogen, Lofer, Austria), secondary anti-mouse and anti-rabbit HRP conjugated antibodies from DAKO (Glostrup, Denmark).

Antibody production:

A C-terminal fragment (residues 475 to 606) was generated by PCR and cloned into the NcoI/XhoI sites of pGEX-SG for bacterial expression and purification of a GST tagged C-

terminal fragment of Spindly for affinity purification of rabbit antibodies generated by Gramsch Laboratories (Schwabhausen, Germany) raised against a C-terminal Spindly peptide (residues 583-601).

µLC-ESI-MS/MS:

Alternatively, proteins were eluted from beads in 8 M urea, digested (Wessel and Flugge, 1984) and peptides analyzed using µ-HPLC-ESI-Iontrap-MS. The LC device consisted of the Ultimate µ HPLC pump and column oven, the Switchos µ column-switching device with loading pump and two 10-port valves and the FAMOS µ-Autosampler (LC Packings, Amsterdam, Netherlands). µ-HPLC was performed using a poly(p-methylstyrene-co-1,2-bis(p-vinylphenyl) ethane) based stationary phase (Trojer *et al.*, 2006) under reversed phase conditions with 0,1 % formic acid in water (A) and 0,1% formic acid in 30% acetonitril (B), at a flow rate of 1 µl/min at 40°C. For separation, a linear gradient (50 min from 0% B to 100% B) was applied. For the hyphenation to the mass spectrometer a nanoflow electrospray ionization source from Proxeon (Odense,Denmark) with Pico Tips from New Objective (FS360-20-10, MA, USA) was used. Mass spectrometric data were obtained on the linear ion trap LTQ (Thermo Fisher Scientific Inc., Waltham, MA) operated at 1.4 kV source voltage, 220 °C capillary temperature, 37 V capillary and 94 V tube lens voltage. Data acquisition and interpretation was done with Xcalibur and database searches with BioworksBrowser 3.3.1 SP1 and Sequest (Thermo Fisher).

Electron microscopy:

Cells were subjected to high-pressure freezing, freeze-substitution (with acetone containing 1% w/v OsO_4, 0.2% w/v UO_2^{2+} and optionally 4% H_2O) and epoxy resin embedding as described (Hess, 2007). Serial 100nm-thin sections were viewed with a Philips CM120 transmission electron microscope (FEI, Eindhoven, The Netherlands), and images were

recorded with a MORADA digital camera (Olympus SIS, Münster, Germany). Contrast and brightness of the images were optimized by using gray scale adjustment and high-pass filtering with Adobe Photoshop software, version 9.

Reference List

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Hess,M.W. (2007). Cryopreparation methodology for plant cell biology. Methods Cell Biol *79*, 57-100.

Trojer,L., Lubbad,S.H., Bisjak,C.P., and Bonn,G.K. (2006). Monolithic poly(p-methylstyrene-co-1,2-bis(p-vinylphenyl)ethane) capillary columns as novel styrene stationary phases for biopolymer separation. J. Chromatogr. A *1117*, 56-66.

Wessel,D. and Flugge,U.I. (1984). A method for the quantitative recovery of protein in dilute solution in the presence of detergents and lipids. Anal. Biochem. *138*, 141-143.

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Supplementary Table I: Summary of mass spectrometry analysis of CCDC99 associated proteins:

			MALDI TOF exp. n°1		MALDI TOF exp. n°2		LC-MSMS exp. n°1		LC-MSMS exp. n°2	
Name	Accession n°	Molecular mass	Coverage	Expect	Coverage	Expect	Coverage	P (pro)	Coverage	P(pro)
KNTC1/Rod	P50748	253211	7%	0.88	15%	3.2e-10	22.5%	1.06e-12		
ZW10	O43264	89628	18%	1.2e-05	27%	3.8e-06	15.9%	1.52e-12		
ZWILCH	Q9H900	67171					27.70%	6.41e-08	43.5%	5.7e-09
CCDC99	Q96EA4	70120	55%	1.3e-07			44.3	1.42e-13	74.5%	2.00e-09

Supplementary Figure legends:

Supplementary Figure 1: **Characterisation of human Spindly. (A) Spindly is a mitotic phosphoprotein.** FLAG-tagged Spindly immunoprecipitated from nocodazole treated HeLa cells, analysed by SDS-PAGE and cCBB stained was excised from the gel, in-gel digested with trypsin and analysed by MS. (left panel) MALDI TOF/TOF peptide mass fingerprint showing prominent neutral loss of ~98 Da, corresponding to H_3PO_4. (right panel) ESI-CID tandem MS spectrum indicating S515 as the phosphorylated residue. Spectral data were analysed with Sequest and gave an XCorr of 4.64 and a peptide probability of 5.24e^-7. **(B) Spindly is part of a large protein complex.** Top panels: ACA34: 1.5 mg of mitotic HeLa cell lysate was fractionated by gel filtration on a calibrated Tricorn 10/600 ACA34 column and fractions probed for Spindly, ZW10, Rod, and α-tubulin. Fractions containing the peaks of molecular weight standards are indicated. Bottom panels: Superose 6: Mitotic Hela cell lysate was fractionated on a calibrated Tricorn 10/600 60 Superose 6 column and fractions 2-11 probed for Spindly, ZW10, Rod and Dynamitin. Molecular weight standards are indicated. **(C) Spindly co-localises with ZW10 on kinetochores.** HeLa cells stained for DNA (grey), kinetochores (CREST, blue), Spindly (red) and ZW10 (green). Images were taken on a Leica SP5 microscope. Size bar=5 μm. **(D) In mitosis, Spindly localises to kinetochores and the mitotic spindle but does not associate with anaphase chromosomes.** HeLa cells were fixed using cold methanol and stained for Spindly using a polyclonal mouse antiserum (Abnova, green) and DNA (Hoechst33342, red). In early prometaphase Spindly stains kinetochores, while in late prometaphase, signals are shifted to the mitotic spindle and the spindle poles. No Spindly signal could be detected on ana/telophase chromsomes. **(E) Knockdown of ZWINT1 depletes Spindly from kinetochores.** HeLa cells transfected with control, ZWINT1 and Spindly siRNA for 36 h stained for DNA, kinetochores, ZWINT1 and Spindly. Size bar=5μm. **(F) Knockdown of ZW10 depletes Spindly from kinetochores.** Hela cells transfected with

control, ZW10 and Spindly siRNA stained for DNA, kinetochores, ZW10 and Spindly. Size bar=5μm. **(G) Spindly levels are reduced in cells lacking ZW10.** HeLa cells were transfected with 50nM control, ZW10, ZWINT1 and KNTC1/Rod specific siRNA for 48 h. Total cell lysates were fractionated by denaturing gel electrophoresis, blotted for Spindly, ZW10, Zwint1, Rod, GAPDH and visualised using ECL.

Supplementary Figure 2: **Spindly controls dynein levels at kinetochores.** (A) Efficiency of Spindly knockdown in human cells. Total cell lysates obtained 48 h after siRNA transfection (50 nM) were analysed by immunoblotting for Spindly and GAPDH. (B) Spindly signals at KCs were determined by immunofluorescence staining and confocal imaging. Kinetochore Spindly signals were normalised to co-stained CENP-F signals (control n=4, Spindly RNAi n=20). (C) HeLa cells were transfected with control or Spindly specific siRNAs for 36 h, fixed with methanol and stained for DNA, CREST, dynein heavy chain 1 (DHC1) and dynein intermediate chain 1 (DIC1). Deconvoluted z-stacks of representative cells are shown. Size bar is 10μm.

Supplementary Figure 3: **Spindly localisation to kinetochores is controlled by microtubule attachment and tension.** HeLa cells were first treated with 20 μM MG132 for 4 h, followed by 2 h treatment with either solvent, 1 μM nocodazole or 1 μM taxol in the absence (left three columns) or presence of 2μM ZM447439 before methanol fixation and immunostaining for DNA, kinetochores (CREST), microtubules (tubulin) and Spindly. Arrows indicate individual kinetochores. Size bar=10 μm. Intensity scale is the same for all images. Background corrected levels of Spindly on kinetochores were determined from a total of 875 kinetochores in 3 different experiments and plotted as relative levels to the maximum signal in nocodazole treated cells.

Supplementary Figure 4: **Spindly RNAi causes a MAD2-dependent mitotic arrest. (A,B) Spindly RNAi causes effective protein knockdown. (A) Spindly RNAi delays progression through mitosis.** Selected time frames of movies analysed to determine length of mitosis of HeLa and U2OS cell transfected with 50 nM control or Spindly siRNA for 48 h. Time=h:min. (B) Length of mitosis was determined as the duration (min) from chromosome condensation until decondensation in HeLa and U2OS transfected with 50 nM control or Spindly siRNA for 48 h. (C) **MAD2 is required for Spindly RNAi induced arrest in mitosis.** H2B-GFP HeLa cells were transfected with 50nM Spindly siRNA alone or together with MAD2-specific siRNA and images taken 48 h after transfection. Arrows indicate mitotically arrested cells in Spindly RNAi cells and telophase cells with lagging chromosomes and micronuclei in Spindly+MAD2 RNAi cells.

Supplementary Figure 5: **Normal kinetochore formation in Spindly RNAi cells.** Electron microscopy of kinetochores in control (A,B,C) and Spindly-RNAi Hela cells (D,E,F). A: Control HeLa metaphase plate with bundles of microtubules running towards a kinetochore (arrow); scale bar=200 nm. B, D: Overviews show a normal metaphase plate in B and a typical Spindly-RNAi phenotype in D; arrows point to the selected kinetochore shown in A,C,E,F; scale bars=5 µm; C,E: Details of the kinetochore from A, F, respectively; scale bars=200 nm. F: Spindly-RNAi Hela showing apparently intact kinetochore (arrow) with attached microtubules; scale bar=200 nm.

Supplementary Figure 6: **Analysis of the ZWINT1-ZW10-Spindly-Dynein pathway in chromosome congression and duration of mitosis.** Hela cells expressing histone H2B-GFP were transfected with 50nM of siRNAs targeting either one or two mRNAs of the ZWINT-1-ZW10-Spindly-DHC pathway. Transfected cells were monitored by live cell fluorescence imaging 48 h after transfection for 12 h. Size bar=10 µm. Time=h:min.

Supplementary Figure 7: **Visualisation of kinetochore stripping of ZW10.** U2OS cells stably expressing YFP-ZW10 were imaged on a Leica SP5 confocal microscope adapted for live cell imaging. Images were taken using a 63x HCX PL APO lambda blue, NA1.4 objective and 30 s time interval. Arrows indicate fibrillar YFP signal moving from kinetochores towards the spindle poles in late prometaphase. Shown are frames of movie 1 available online.

Legends to movies supplied online:

Supplementary Movie 1: Spindly RNAi phenotype in HeLa-H2B-GFP cells.

This movie shows a representative HeLa-H2B-GFP cell treated with Spindly specific siRNAs. Images were taken every 30 sec using a Zeiss 40x Plan NeoFluar NA0.75 objective on a Zeiss Axiovert200M microscope driven by Metamorph and a CoolSnap*fx* camera. Time is shown in hours and minutes. Scale bar = 10 μm.

Supplementary Movie 2: Control mitosis in HeLa-H2B-GFP cells

This movie shows a high resolution imaging of a representative HeLa-H2B-GFP control cell during mitosis. Images were taken every 30 sec using a Zeiss 40x Plan NeoFluar NA0.75 objective on a Zeiss Axiovert200M microscope driven by Metamorph and a CoolSnap*fx* camera. Time is shown in hours and minutes. Scale bar = 10μm.

Supplementary Movie 3: Spindly RNAi phenotype in U2OS H2B-GFP-Cherry-tubulin cells.

This movie shows a representative U2OS-H2B-GFP/mCherry α-tubulin cell treated with Spindly specific siRNAs. Images were taken every 5 minutes using a Zeiss 40x Plan NeoFluar NA0.75 objective on a Zeiss Axiovert200M microscope driven by Metamorph and a CoolSnap*fx* camera over a time course of more than 10 hours. Time is shown in hours and minutes. Scale bar = 10 μm.

Supplementary Movie 4: Control mitosis in U2OS cells H2B-GFP-Cherry-tubulin cells.

This movie shows a representative U2OS-H2B-GFP/mCherry α-tubulin control cell during mitosis. Images were taken every 5 minutes using a Zeiss 40x Plan NeoFluar NA0.75 objective on a Zeiss Axiovert200M microscope driven by Metamorph and a CoolSnap*fx*

camera over a time course of more than 12 hours. Time is shown in hours and minutes. Scale bar = 10 μm.

Supplementary Movie 5: CENP-E RNAi phenotype in HeLa-H2B-GFP cells.

This movie shows imaging of HeLa-H2B-GFP cell treated with CENP-E specific siRNAs. Images were taken every 2 min using a Zeiss 40x Plan NeoFluar NA0.75 objective on a Zeiss Axiovert200M microscope driven by Metamorph and a CoolSnap*fx* camera. Time is shown in hours and minutes. Scale bar = 20 μm.

Supplementary Movie 6: NUF2-RNAi phenotype in HeLa-H2B-GFP cells.

This movie shows imaging of HeLa-H2B-GFP cells treated with NUF2 specific siRNAs. Images were taken every 2 min using a Zeiss 40x Plan NeoFluar NA0.75 objective on a Zeiss Axiovert200M microscope driven by Metamorph and a CoolSnap*fx* camera. Time is shown in hours and minutes. Scale bar = 10 μm.

Supplementary Movie 7: Metaphase arrest induced by the proteasome inhibitor MG-132.

This movie shows imaging of HeLa-H2B-GFP cells treated with 20 μM MG132. Images were taken every 2 min using a Zeiss 10x Plan NeoFluar NA0.30 objective on a Zeiss Axiovert200M microscope driven by Metamorph and a CoolSnap*fx* camera. Time is shown in hours and minutes. Scale bar = 20 μm.

Supplementary Movie 8: Metaphase arrest induced by low amounts of nocodazole.

This movie shows imaging of a representative HeLa-H2B-GFP cell treated with 25nM nocodazole. Images were taken every 2 min using a Zeiss 40x Plan NeoFluar NA0.75

objective on a Zeiss Axiovert200M microscope driven by Metamorph and a CoolSnap*fx* camera. Time is shown in hours and minutes. Scale bar = 10 μm.

Supplementary Movie 9: Kinetochore movements in U2OS CenpB-YFP control cells.

This movie shows imaging of U2OS-CENP-B-YFP cells transfected with 50nM control siRNAs for 36 h. Images were taken every 20s using a Zeiss 63x ApoPlan oil immersion, NA1.4 objective on a Zeiss Axiovert200M microscope driven by Metamorph and a Cascade 2 camera. Time is shown in Min:sec. Scale bar = 10 μm.

Supplementary Movie 10: Kinetochore movements in U2OS CenpB-YFP Spindly RNAi cells.

This movie shows imaging of U2OS-CENP-B-YFP cells transfected with 50nM Spindly siRNAs for 36h. Images were taken every 20s using a Zeiss 63x ApoPlan oil immersion, NA1.4 objective on a Zeiss Axiovert200M microscope driven by Metamorph and a Cascade 2 camera. Time is shown in Min:sec. Scale bar = 10μm.

Supplementary Movie 11: Kinetochore movements in U2OS CenpB-YFP ZW10 RNAi cells.

This movie shows imaging of U2OS-CENP-B-YFP cells transfected with 50nM ZW10 siRNAs for 36 h. Images were taken every 20 s using a Zeiss 63x ApoPlan oil immersion, NA1.4 objective on a Zeiss Axiovert200M microscope driven by Metamorph and a Cascade 2 camera. Time is shown in Min:sec. Scale bar = 10 μm.

Supplementary Movie 12: Kinetochore movements in U2OS CenpB-YFP Spindly/ZW10 RNAi cells.

This movie shows imaging of U2OS-CENP-B-YFP cells transfected with 50 nM control siRNAs for 36 h. Images were taken every 20 s using a Zeiss 63x ApoPlan oil immersion, NA1.4 objective on a Zeiss Axiovert200M microscope driven by Metamorph and a Cascade 2 camera. Time is shown in Min:sec. Scale bar = 10 μm.

Supplementary Movie 13: Live cell imaging of YFP-ZW10 proteins dynamics.

This movie shows high resolution imaging of a representative U2OS-YFP-ZW10 cell during late prometaphase. Images were taken every 30 sec using a HCX PL APO lambda blue 63X, NA 1.4 oil UV objective on a Leica TCS-SP5 DMI6000 confocal microscope. Time is shown in minutes and seconds. Scale bar = 10 μm.

Supplementary Movie 14: Spindly overexpression in HeLa-H2B-GFP cells.

This movie shows a representative mRFP positive HeLa-H2B-GFP cell transfected with FLAG-tagged Spindly expression plasmid during mitosis. Images were taken every 5 minutes using a Zeiss 40X Plan NeoFluar NA 0.75 objective on a Zeiss Axiovert200M microscope driven by Metamorph and a Cascade 2 camera over a time course of more than 10 hours. Time is shown in hours and minutes. Scale bar = 10 μm.

Supplementary Movie 15: ΔQQ Spindly mutant overexpression in HeLa-H2B-GFP cells.

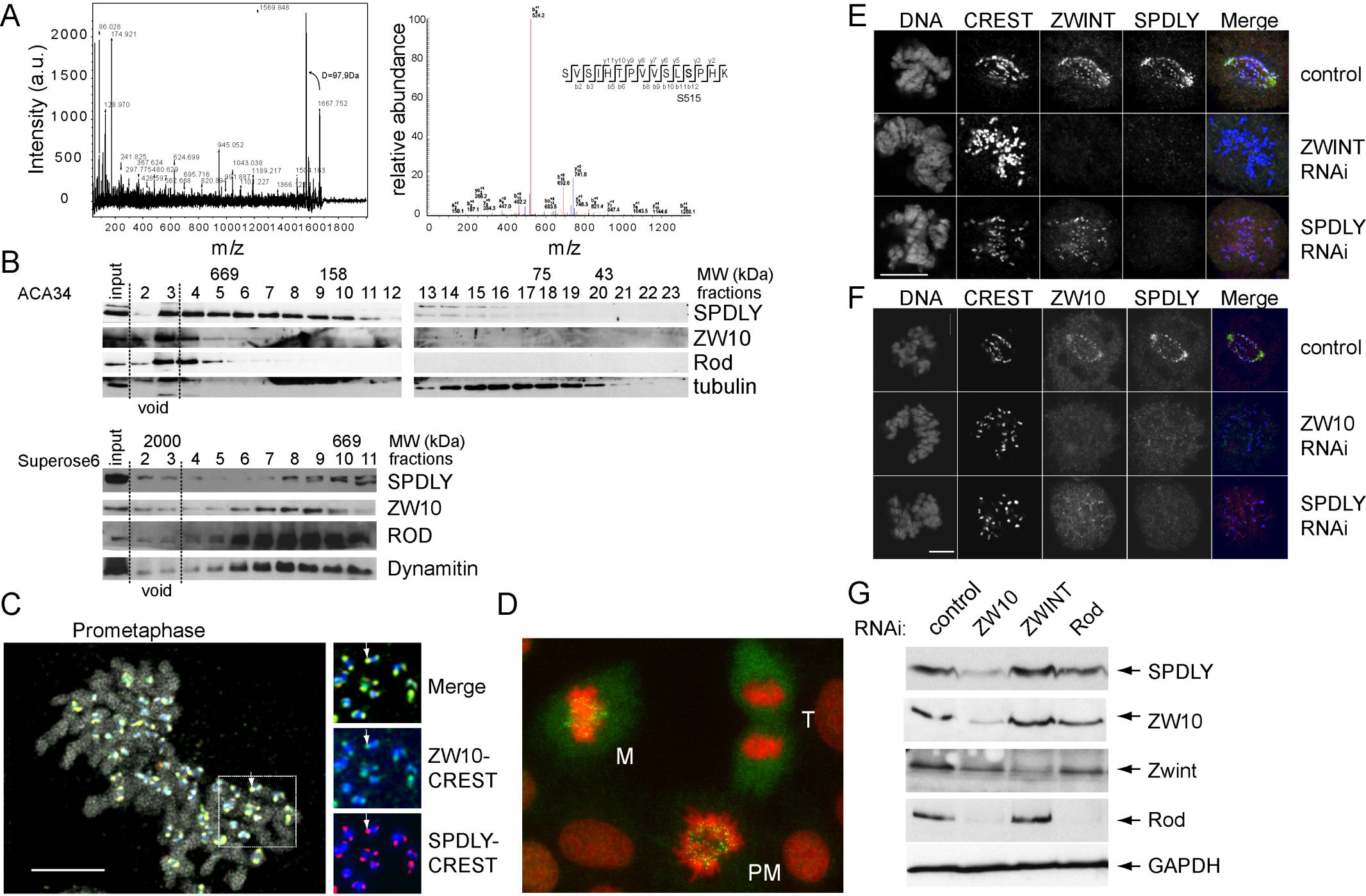
This movie shows a representative mRFP positive HeLa-H2B-GFP cell transfected with ΔQQ Spindly. Images were taken every 5 minutes using a Zeiss 40X Plan NeoFluar NA 0.75 objective on a Zeiss Axiovert200M microscope driven by Metamorph and a Cascade 2 camera over a time course of more than 10 hours. Time is shown in hours and minutes. Scale bar = 10 μm.

Supplementary Movie 16: NΔ253 Spindly mutant overexpression in HeLa-H2B-GFP cells.

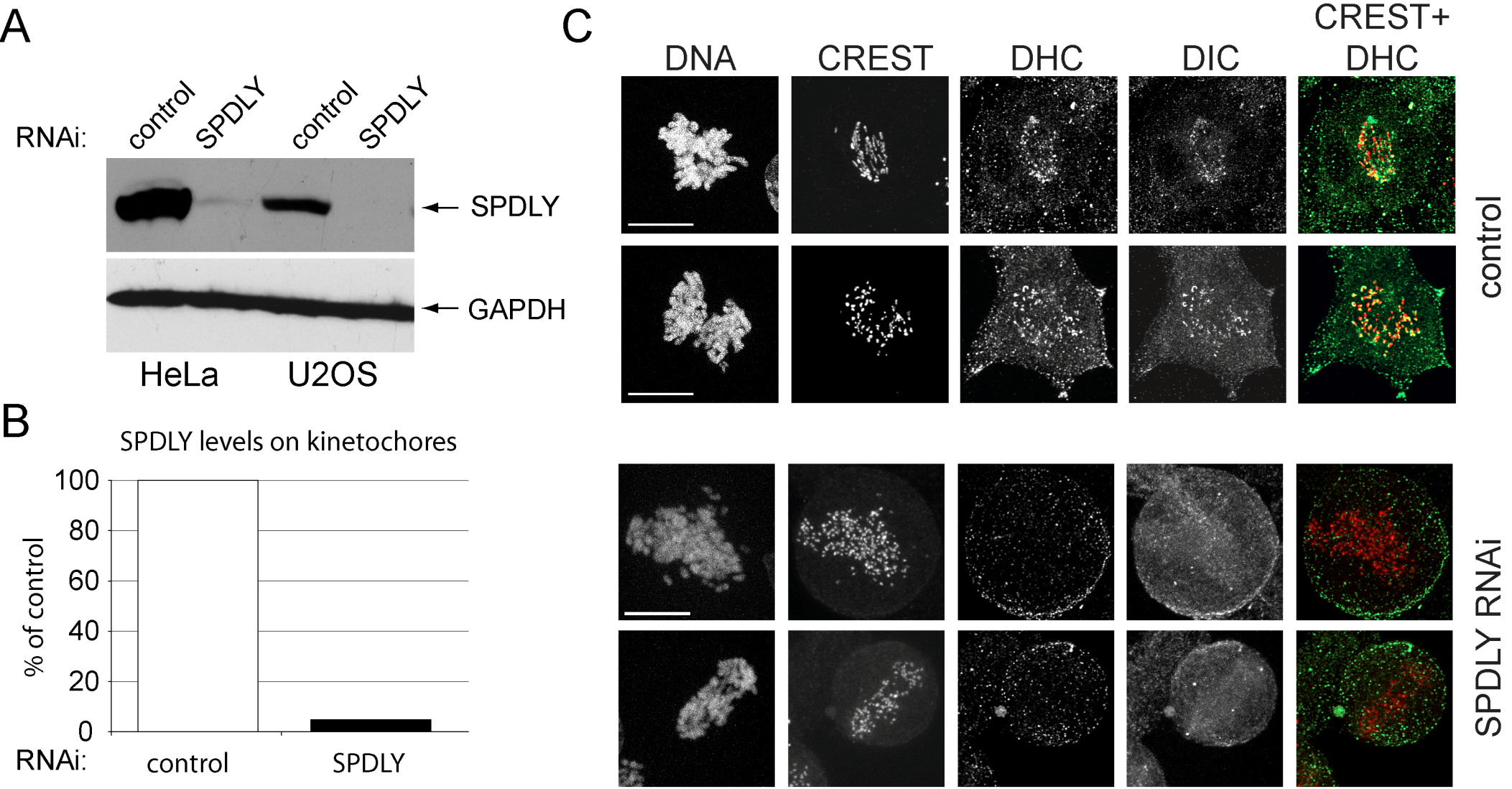
This movie shows a representative mRFP positive HeLa-H2B-GFP cell transfected with NΔ253 Spindly expression plasmid during mitosis. Images were taken every 5 minutes using a Zeiss 40X Plan NeoFluar NA 0.75 objective on a Zeiss Axiovert200M microscope driven by Metamorph and a Cascade 2 camera over a time course of more than 10 hours. Time is shown in hours and minutes. Scale bar = 10 μm.

Supplementary Movie 17: ΔSB Spindly mutant overexpression in HeLa-H2B-GFP cells.

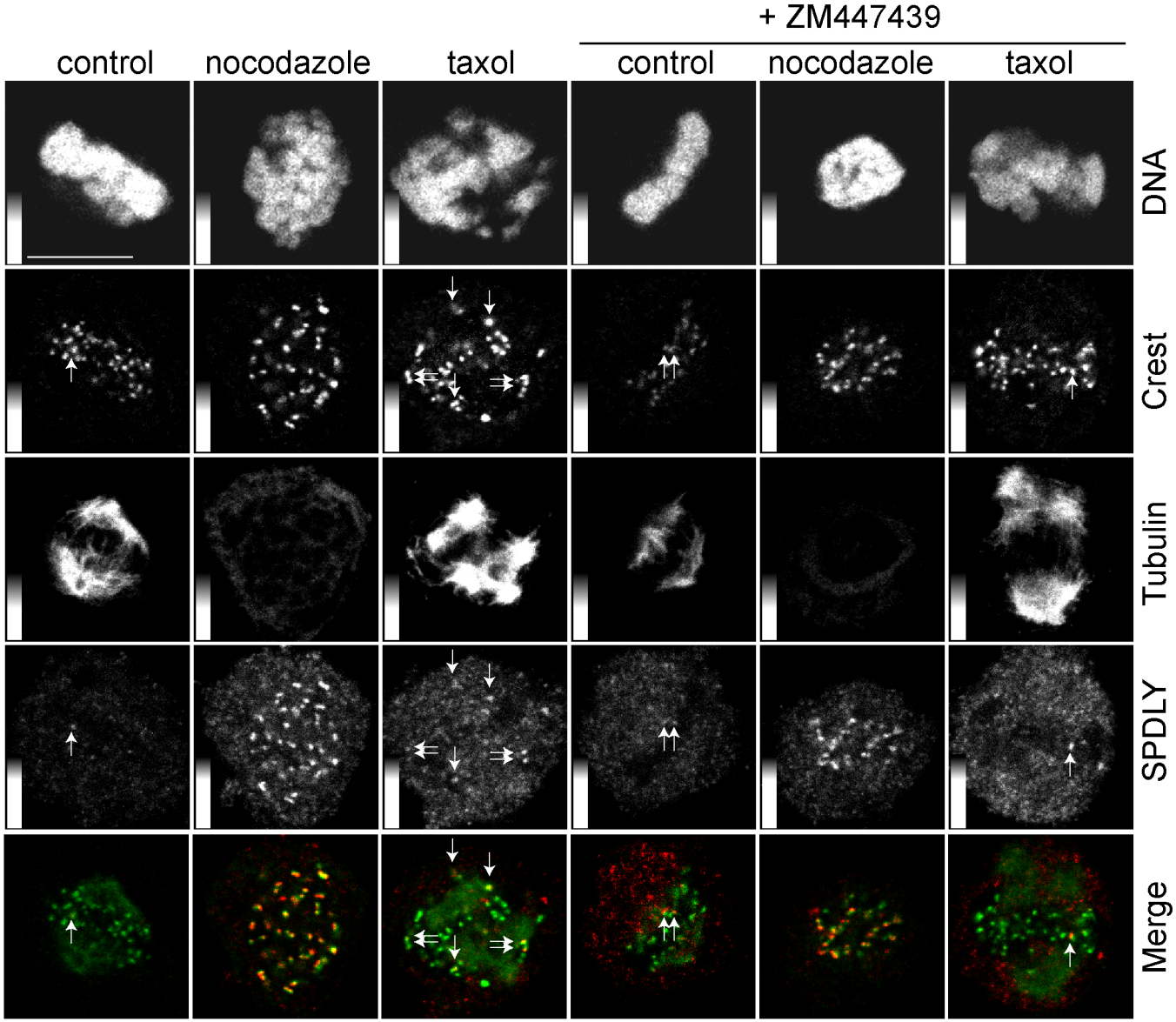
This movie shows mitosis of a representative mRFP positive HeLa-H2B-GFP cell transfected with a Spindly ΔSB expression plasmid. Images were taken every 5 minutes using a Zeiss 40X Plan NeoFluar NA 0.75 objective on a Zeiss Axiovert200M microscope driven by Metamorph and a Cascade 2 camera over a time course of more than 10 hours. Time is shown in hours and minutes. Scale bar = 10 μm.

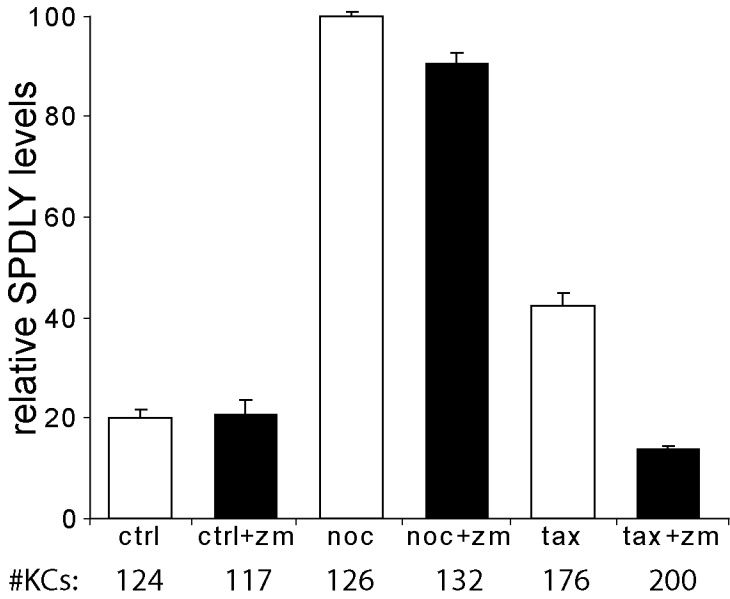


Supplementary Figure 1

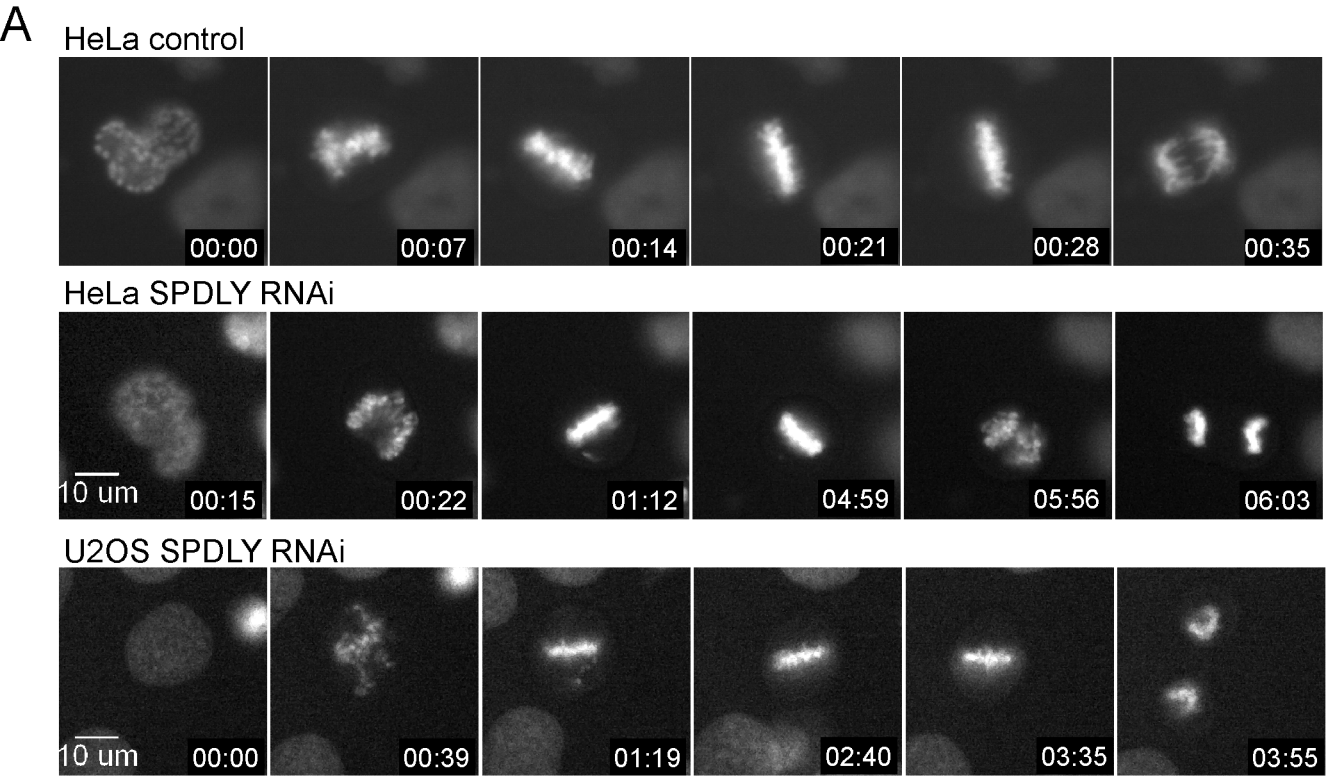


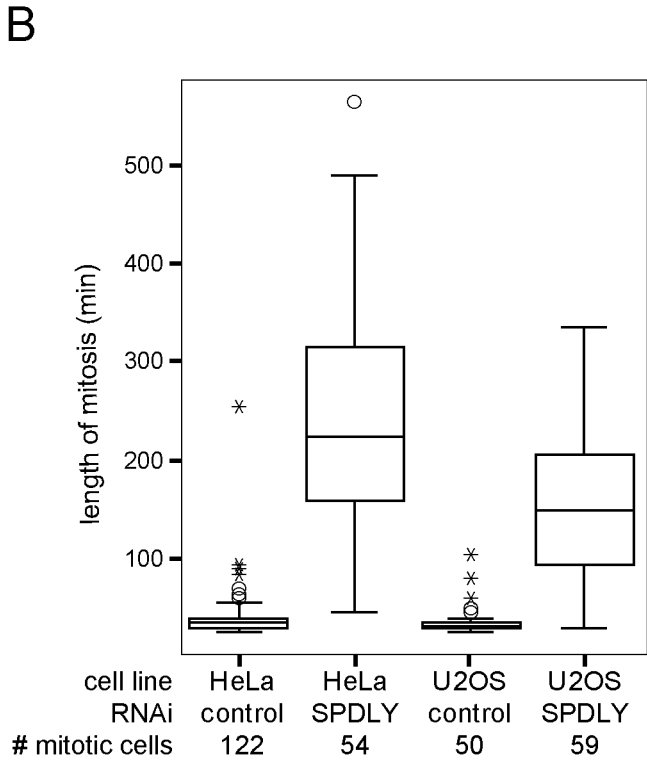
Supplementary Figure 2

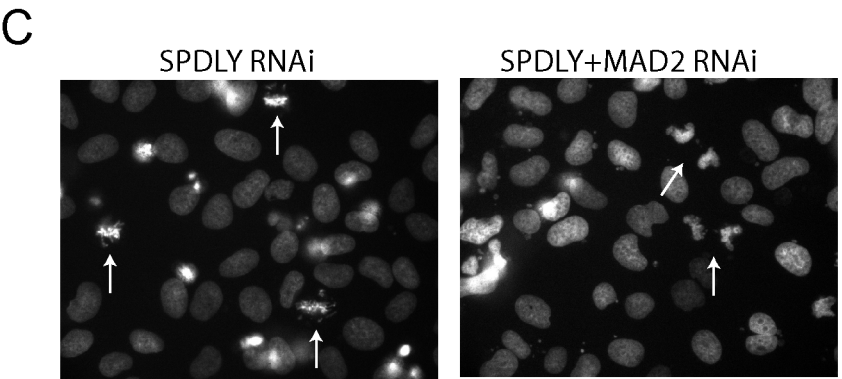




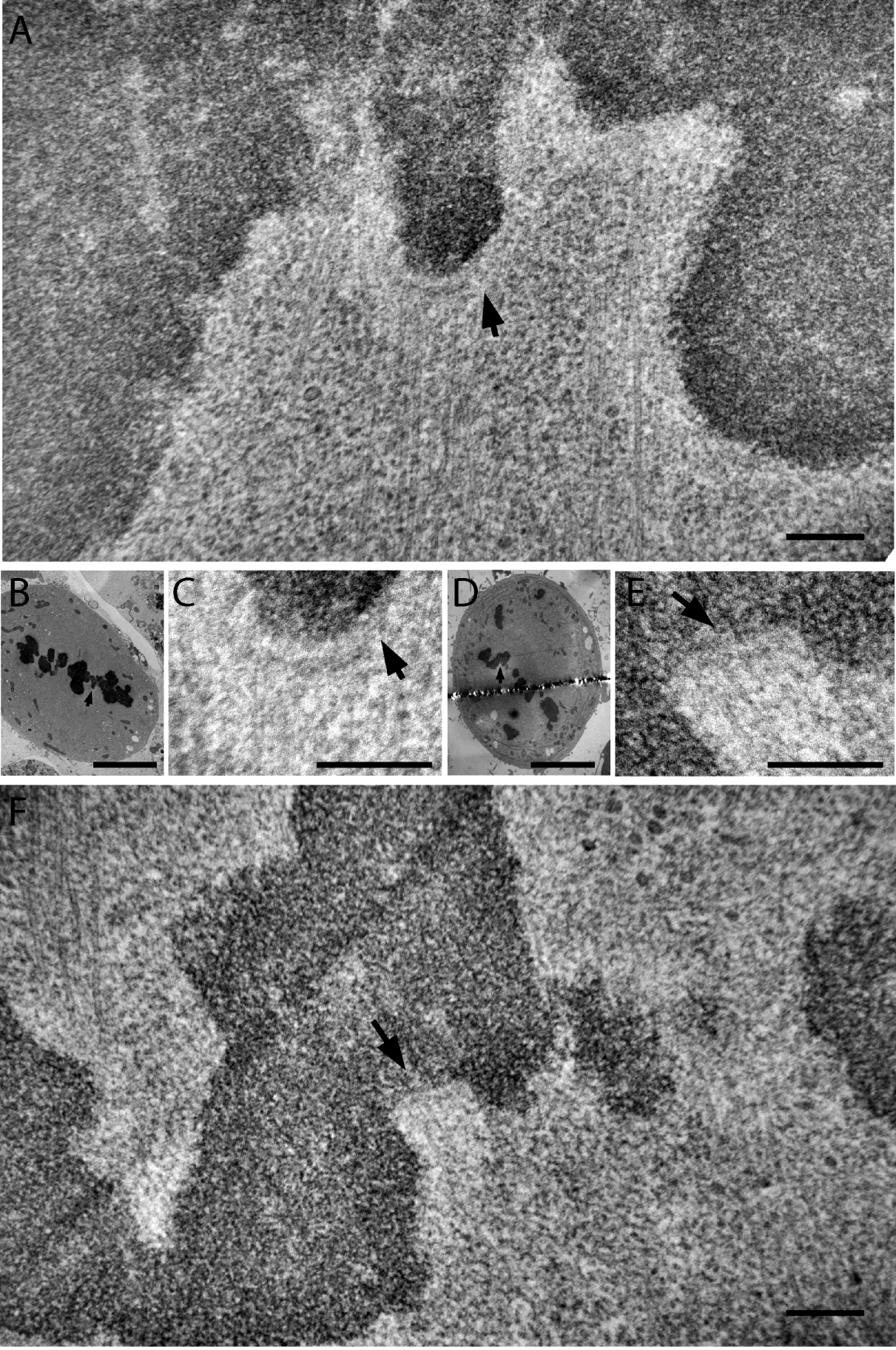
Supplementary Figure 3



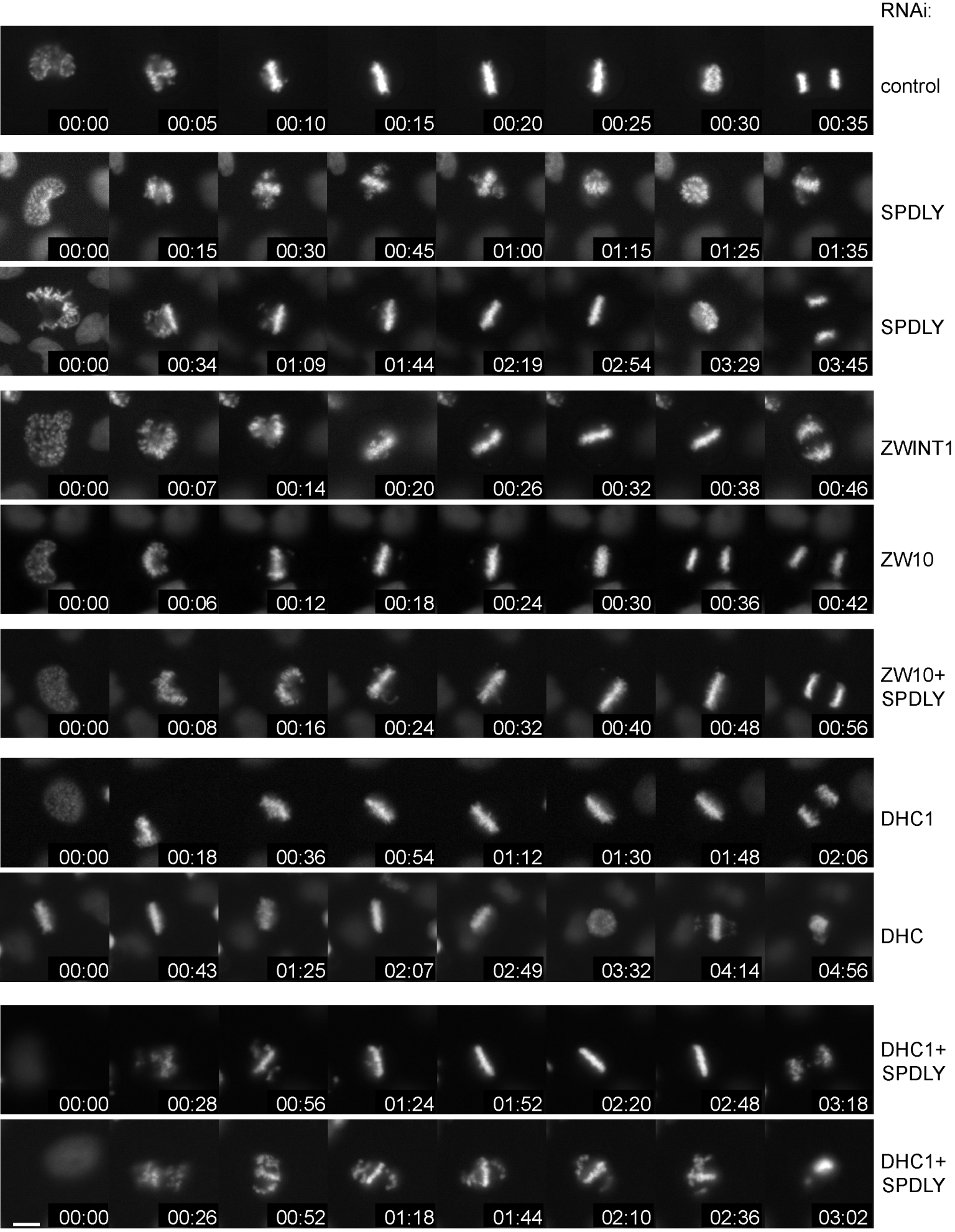




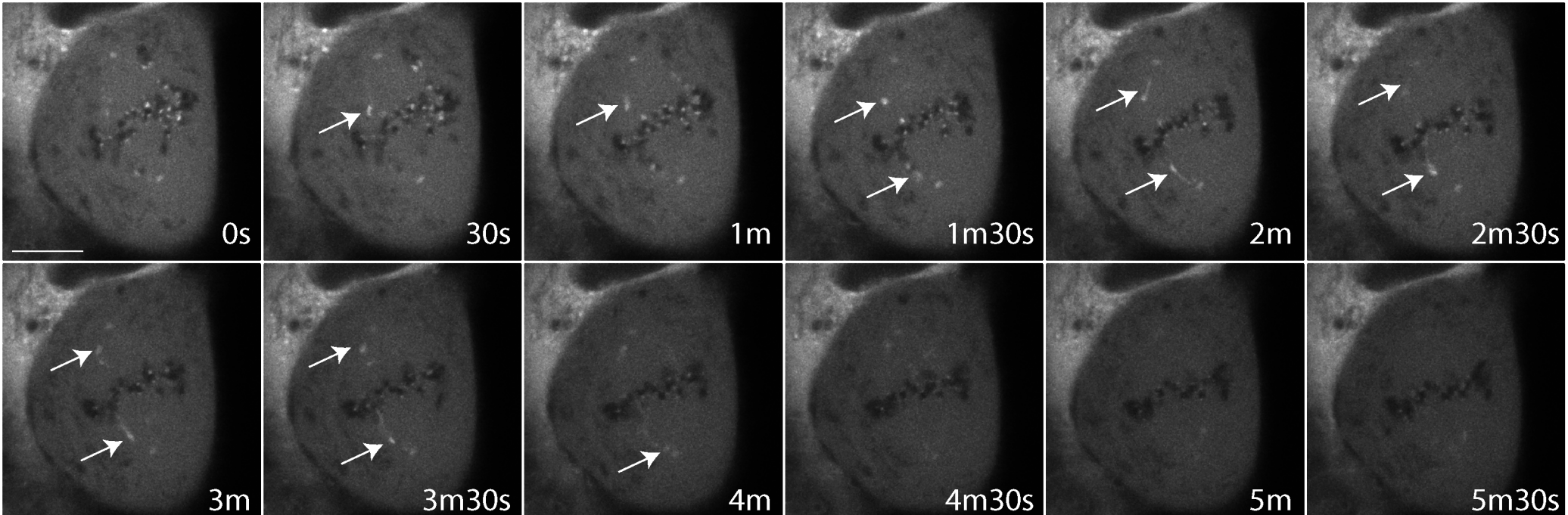
SUPPLEMENTARY FIGURE 4



Supplementary Figure 5



SUPPLEMENTARY FIGURE 6



SUPPLEMENTARY FIGURE 7