Supplemental Information

Experimental Procedures

Bioinformatics identification of C13ORF3 as a putative kinetochore-associated protein

C13ORF3 was identified as a potential kinetochore-related gene by a novel bioinformatics methodology that entails a gene-centric meta-analysis (GMA) of microarray data [1]. A total of 3,651 human 2-color microarray datasets were downloaded for analysis from the Gene Expression Omnibus (GEO) repository [2]. Briefly, the GMA first identified genes co-expressed with C13ORF3 and ranked them by how consistently and frequently they were co-expressed in the same direction as C13ORF3 (i.e., upregulated and downregulated together), weighting the relevance of each gene-gene association with a combination of mutual information (i.e., to account for the differences in the relative frequency by which genes are differentially expressed), the total number of times each gene pair was co-expressed and the "purity" of the pattern by which they were co-expressed. (If a gene pair always change their expression in the same direction, the "purity" of their pattern is 100%.)

Once each of the genes co-expressed with C13ORF3 were identified and ranked, the top 20 were sent for a literature-based analysis of the published associations they had in common. To do this, software called IRIDESCENT was used [3-5]. Briefly, IRIDESCENT uses a term thesaurus to recognize when biological "objects" (e.g. genes, diseases, phenotypes, chemicals, etc) co-occur within a MEDLINE abstract. The thesaurus is constructed using popular and freely available sources for object names (e.g. Entrez gene, OMIM, ChemID database, etc) [4]. At the time of this study, the IRIDESCENT database of associations was constructed using 18,438,436 MEDLINE records, 55% of which had abstracts with the rest only having titles. IRIDESCENT identified many different aspects of the cell division cycle as a strongly associated commonality of these genes, and the kinetochore as one of the highest-scoring associations. The IRIDESCENT analysis and association with the kinetochore was corroborated with a Gene Ontology Enrichment analysis ($p < 0.03$) via DAVID [6].

Cell Culture

Hela cells were grown in DMEM supplemented with 10% fetal bovine serum, 20 mM HEPES, 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, 2 mM L-glutamine, and 0.1 mg/ml penicillin/streptomycin. The concentrations of drugs used were Nocodazole, 330 nM, Taxol, 10 μM, ZM447439, 25 μM and MG132, 25 μM.

RNAi Tools and Screening

Short hairpin RNA expression vectors were made by using RNAi-Ready pSIREN-DNR-DsRed-Express Vector (Clontech Laboratories, Inc.) following the manufacturer's manual. In brief, two target regions of 19 nucleotides were chosen for the knockdown of gene expression. Target sequences were selected by using a combination of RNAi Designer tools from Clontech (http://bioinfo.clontech.com/rnaidesigner/frontpage.jsp) and database searches of the RNAi Consortium shRNA Library at the Broad Institute (http://www.broad.mit.edu/rnai/trc/lib). The target sequences selected for Ska3 were 5' GGTACATCGTATCCCAAGT 3' and 5' ACACGAGCAAGAAGCCATT 3'. Complementary oligonucleotides were synthesized (Integrated DNA Technologies) to contain restriction sites, target sequence, hairpin loop, and antisense sequence. Oligonucleotides were then annealed, ligated into the vector, and transformed into DH5 alpha competent cells (Invitrogen). Individual colonies were screened and verified by DNA sequencing.

Hela cells stably transfected with GFP fused to histone 2B (H2B) were used for screening of shRNA plasmids. Cells were grown on 8 chambered glass slides (Lab-Tek) in DMEM with 10% FBS in 5% CO_2 at 37ºC. Hela cells were transiently transfected using FuGENE HD (Roche) according to the manufacturer's directions. Live imaging experiments were started 24 hours after transfection. Time-lapse fluorescence images were collected using 20x objectives using a Nikon Eclipse TE2000-E inverted microscope equipped with Nikon's Perfect Focus System (PFS) and a enclosed stage incubator and a Roper Coolsnap HQ camera and Nikon software or with a Zeiss Axiovert 200M inverted microscope equipped with a stage heater, air curtain, and a Hamamatsu ORCA-ER camera and Metamorph software (MDS Analytical Technologies). Images were captured every 4 – 5 minutes for 18 – 24 hours. Time lapse videos were assembled using Metamorph software.

Small interfering RNA (siRNA) duplexes targeting Ska3 were purchased from Dharmacon/Thermo Scientific (ON-Target plus SMART pool, L-015700-00-0005). The target sequences are as follows: 5' GGAAGAGCCCGUAAUUGUA 3', 5' GAUCGUACUUCGUUGGUUU 3', 5' AAUCCAGGCUCAAUGAUAA 3', and 5' CAUCGUAUCCCAAGUUCUA 3'. Small interfering RNA duplexes targeting Shugoshin/Sgo1 (Shugoshin, 5' AAGAUAUCAUCCUACAGCUGAUU 3') and siRNA duplexes targeting BubR1 (5' GUCUCACAGAUUGCUGCCUUU 3') were synthesized (Dharmacon/Thermo Scientific). Hela cells or Hela H2B-GFP cells were transfected with the siRNA's targeting Ska3, BubR1, and/or Shugoshin at 25 - 100 nM using Lipofectamine RNAiMax (Invitrogen) according to the manufacturer's recommendations. Live cell time-lapse fluorescence imaging was performed as described above starting 24 – 30 hours post transfection. For analysis of mitotic progression in time-lapse images, metaphase alignment was defined as the alignment of all visible chromosomes at the spindle midzone observed in three successive image acquisitions encompassing an eight minute period.

Ska3 Cloning and Anti-Ska3 Antibody Production

A clone containing full length Ska3 (C13orf3) was obtained from the ATCC (Mammalian Gene Collection/I.M.A.G.E. Clone 993981). Ska3 was amplified by high fidelity PCR and cloned by restriction digest into pET28a bacterial expression plasmid (Novagen) and into pEGFP-N1 mammalian expression plasmid (Clontech). Clones were verified by sequencing analysis. 6X-Histadine tagged Ska3 protein synthesis was induced in BL21(DE3)pLysS *E.coli* transformed with pET28a-Ska3. Purified 6X-Histadine Ska3 was the immunogen for antibody production in NZW rabbits (Covance Research Products). Anti-Ska3 antibody was affinity purified using beads covalently bound with Ska3 (CNBr-activated Sepharose 4B, GE Healthcare).

Cell Fixation and Immunofluorescence Labeling:

Hela cells growing on glass coverslips were co-fixed/extracted in 1.5% Formaldehyde, 60 mM Pipes, 25 mM Hepes, pH 6.9, 10 mM EGTA, 4 mM $MgSO_4$ (PHEM), 0.5% Triton X-100, and protease inhibitor cocktail (Sigma, P8340) for 15 minutes at room temperature. For swollen-cell fluorescence experiments, mitotic Hela cells were washed in 10 mM Hepes, pH 7.4, 40 mM KCl, 5 mM EGTA, 4 mM $MgSO_4$ by centrifugation. Cells were permeabilized in PHEM, 0.5% Triton X-100, 1 mM DTT, and protease

inhibitor cocktail. Permeabilized cells were centrifuged through a cushion of lysis buffer containing 10% glycerol over poly-L-lysine treated glass coverslips at 1500xg for 10 minutes at 4°C. Pelleted cells were then fixed with 1.5% formaldehyde in PHEM. For cold-calcium lysis experiments, cells grown on coverslips were rinsed in 4°C 0.1 M Pipes, pH 6.95 then placed in 4°C 0.1M Pipes, pH 6.95,1% Triton X-100, 80 uM $CaCl_2$ for 5 minutes. After another rinse, cells were fixed for 15 minutes at room temperature in 2% formaldehyde,0.2% glutaraldehyde, 0.5% Triton X-100, PHEM. For chromosome spreads, mitotic cells were collected from RNAi cultures 48 hours post transfection of siRNA duplexes. At 30 hours post transfection, prior to the addition of nocodazole (in indicated cultures) and the aforementioned 48 hour mitotic cell collection, mitotic cells from RNAi cultures and parallel mock-transfected cultures were removed by pipetting/shake-off and discarded. At this time culture media was replaced with fresh media containing either nocodazole or DMSO (the solvent for nocodazole). Therefore, the assessed group of mitotic cells arrested due to protein depletion examined at 48 hours post siRNA duplex transfection could be appropriately compared to cultures treated with nocodazole alone or to cultures facing a combination of both RNAi-induced protein depletion and nocodazole because mitotic cells in these parallel cultures accumulated during the same time frame. This also insured that all chromosome spreads resulting from mitotic cells treated with nocodazole ± RNAi reflected the state of sister-chromatid pairing in the total absence of spindle formation and microtubule-kinetochore interactions. Collected mitotic cells were swollen in a 1:1 mixture of media and ddH_2O for 20 minutes at 37C, ± nocodazole at 330 ng/ml as indicated in the text. Cells were suspended and washed by centrifugation in 3:1 MEOH:Glacial Acetic Acid and then dropped from a height of at least 45 centimeters onto cleaned glass coverslips. After drying at room temperature, chromosome spreads were stained with Vybrant Green (1:15,000, Invitrogen) in ddH_2O for 5 minutes. Spreads were mounted onto slides with Vectashield (Vector Laboratories) containing 10 mM $MgSO_4$.

For immunolabeling, coverslips were rinsed in 10 mM MOPS, pH 7.4, 150 mM NaCl, 0.05 % Tween-20 (MBST) and blocked for one hour in 20% boiled normal goat serum (BNGS) in MBST or in PBS with 2% BSA. Cells were incubated in primary antibodies diluted in MBST containing 5% BNGS or in PBS containing 2% BSA. Primary antibodies included Rabbit anti-Ska3 (1:600 dilution from immune serum or 1 ug/ml from affinity purified antibody, this study), autoimmune anti-centromere serum (1:800 dilution, Cortex

Biochem), mouse anti-BUB1 (antibody 4B12, 1:100 dilution from Dr. Steve Taylor, University of Manchester), rabbit anti-Mad2 (antibody L317, 1:500 dilution from Dr. Ted Salmon, University of North Carolina), rabbit anti-BubR1 (1:1000 dilution from Dr. Todd Stukenberg, University of Virginia), rat anti-Tubulin (YL1-2, 1:800 dilution from Serotec), rabbit anti-Shugoshin (1:2000 dilution from Dr. Hongtao Yu, University of Texas, Southwestern). Samples on coverslips were washed in MBST or PBS and then incubated with secondary antibodies in MBST containing 5% BNGS or in PBS with 2% BSA. Secondary antibodies (Jackson Immunoresearch) included FITC goat anti-human (1:800), FITC donkey anti-rat (1:800), Cy3 goat anti-mouse (1:1000), and Cy3 goat anti-rabbit IgG (1:800). This was followed by DNA staining with DAPI for 2 min (0.5 µg/ml in water) before mounting in Vectashield containing 10 mM $MgSO_4$.

Three-dimensional fluorescence images were acquired using a Zeiss Axioplan II microscope equipped with 63X (N.A. 1.4) and 100X objectives (N.A. 1.4), a Hamamatsu Orca 2 camera (Hamamatsu Photonics), and Metamorph imaging software (MDS Analytical Technologies). Measurements of fluorescence intensities from acquired images were obtained using Metamorph imaging software and Microsoft Excel. Briefly, regions defining kinetochores were assigned by signals detected using autoimmune centromere antibodies. These regions and larger concentric regions for background determination were used to obtain the kinetochore, BUB1, BUBR1, and MAD2 fluorescence intensities (Ahonen et. Al, Reference 16 in V2 of Ska3 MS). Relative Bub1, BubR1, and Mad2 fluorescence levels were computed from the ratio of their fluorescence intensities to their respective kinetochore fluorescence intensities from the autoimmune centromere antibody. These values are reported after being normalized to prometaphase fluorescence levels in control samples. Measurements of Bub1, BubR1, and Mad2 intensities are derived from a minimum of 200 kinetochores from at least 5 cells per mitotic phase. For determining the amount of tubulin within cold-treated cells, the fluorescence intensity resulting from anti-tubulin antibody staining was calculated. Background fluorescence was removed by determining the average pixel intensity per unit area adjacent to the tubulin fluorescence signals within each cell, multiplying this value by the area containing tubulin fluorescence, and subtracting this result from the tubulin fluorescence intensity.

Western Blotting

Hela cell extracts were prepared by lysis in 20mM Tris-Cl, pH 7.7, 100 mM KCl, 50 mM Sucrose, 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, 0.5% Triton X-100 buffer containing protease inhibitor cocktail (Sigma), 1 mM DTT. Extracts were supplemented with 2 mM $MnCl_2$ and 1000U/ml lambda phosphatase and incubated at 30°C for 5 minutes. Extracts were prepared for electrophoresis by applying sample loading buffer (Invitrogen) containing 50 mM DTT. Proteins were separated with a NuPAGE gel electophoresis system (Invitrogen), transferred to 0.45 um PVDF membrane (Immobilon PVDF, Millipore) via a Genie transfer apparatus (Idea Scientific). Membranes were blocked in 5% Non-Fat Dry Milk (NFDM) or 5% BSA in Tris-buffered saline with 0.05% Tween 20 (TBST) or Phosphate-buffered saline with 0.05% Tween 20 (PBST). Primary antibodies included rabbit anti-Ska3 antibody (this study) and mouse anti-βactin (Abcam). Membranes were washed in TBST or PBST, and then incubated with secondary antibodies TBST or PBST. Secondary antibodies include HRP goat anti-mouse or anti-rabbit antibodies (Jackson Immunoresearch). After washes, membranes were developed using West Pico Chemiluminescent reagent (Pierce) and imaged using a Kodak 4000M imaging station.

Supplemental References

1. Wren, J.D. (2009). A global meta-analysis of microarray expression data to predict unknown gene functions and estimate the literature-data divide. Bioinformatics (in press).
2. Barrett, T., Troup, D.B., Wilhite, S.E., Ledoux, P., Rudnev, D., Evangelista, C., Kim, I.F., Soboleva, A., Tomashevsky, M., and Edgar, R. (2007). NCBI GEO: mining tens of millions of expression profiles--database and tools update. Nucleic Acids Res *35*, D760-765.
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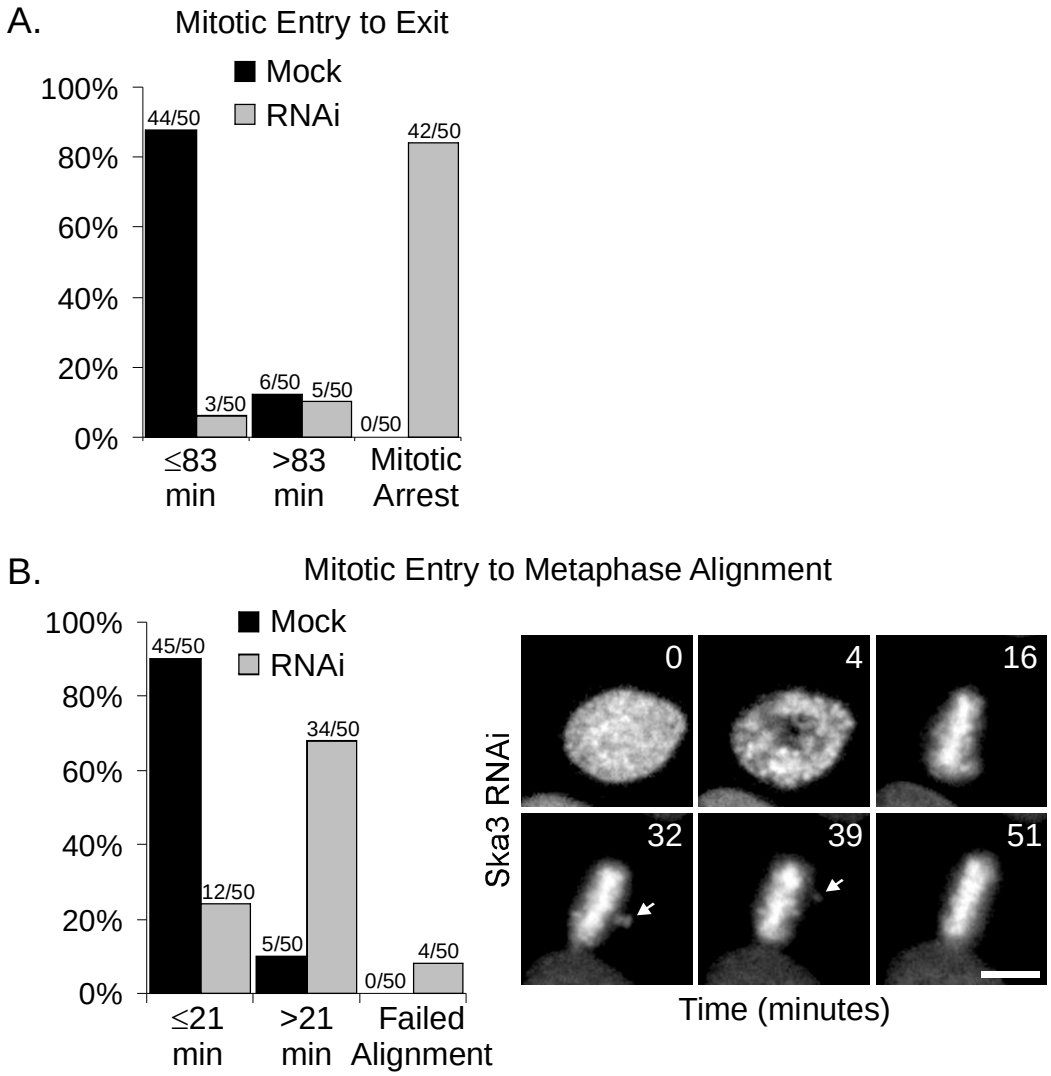
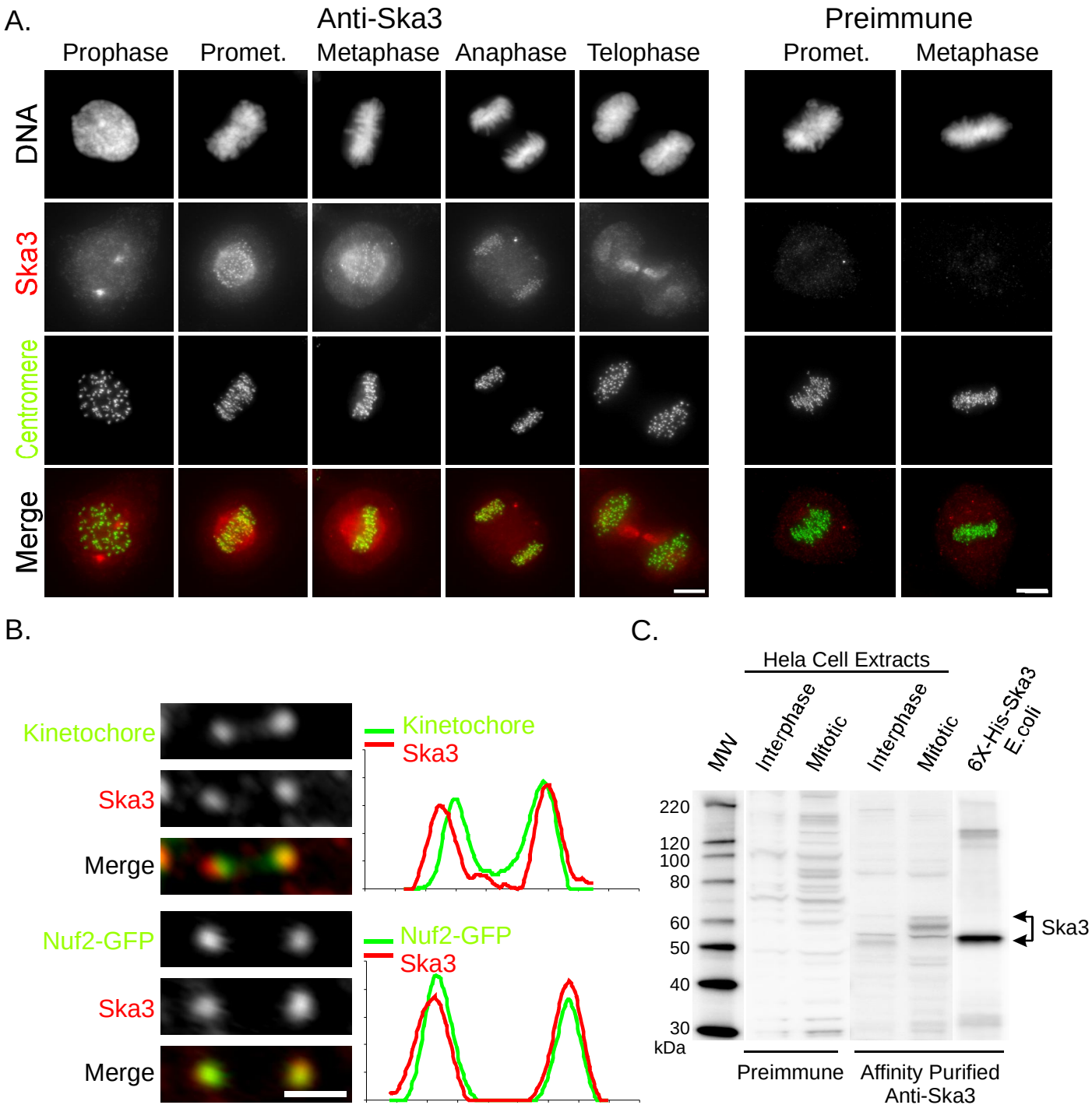
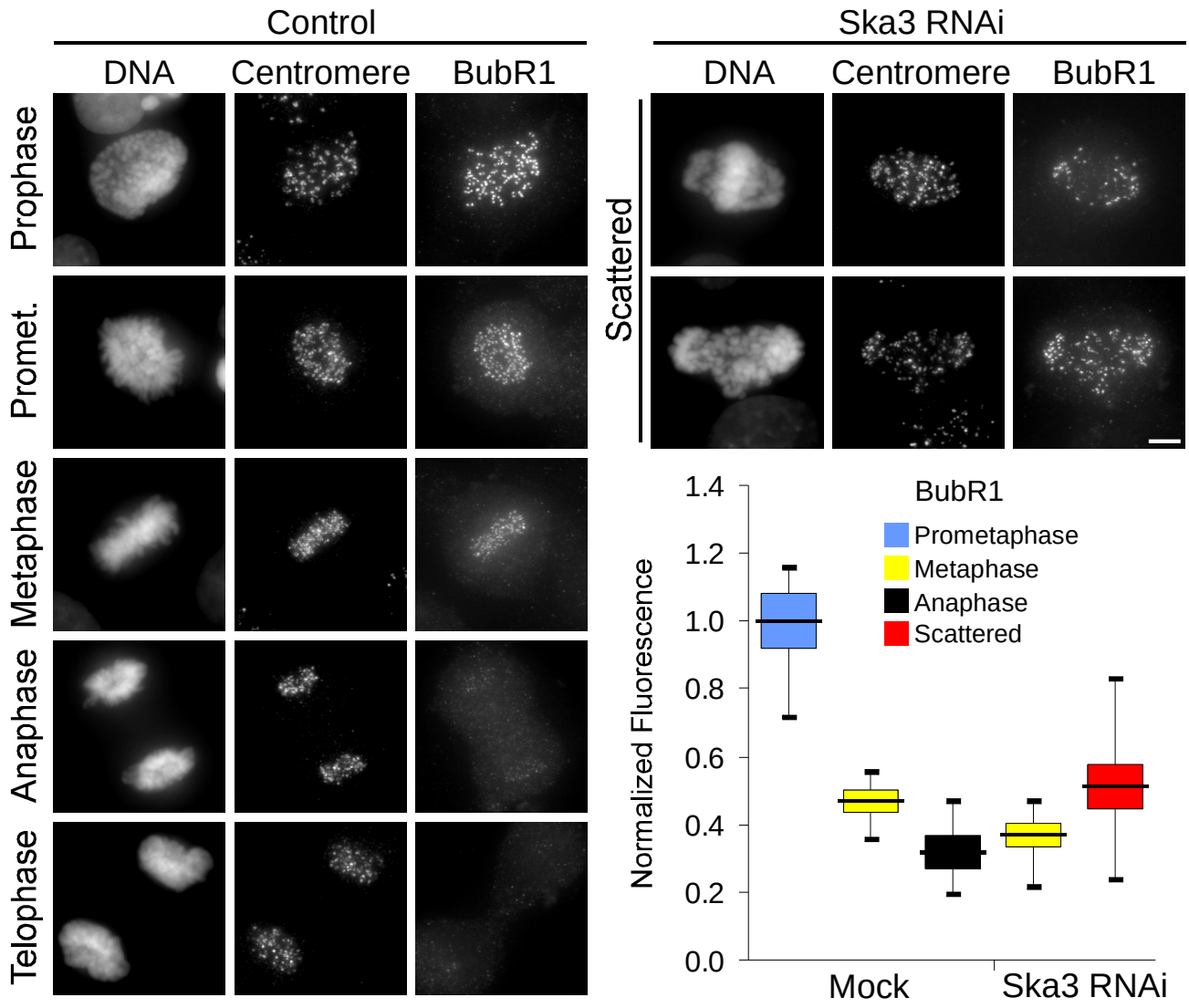


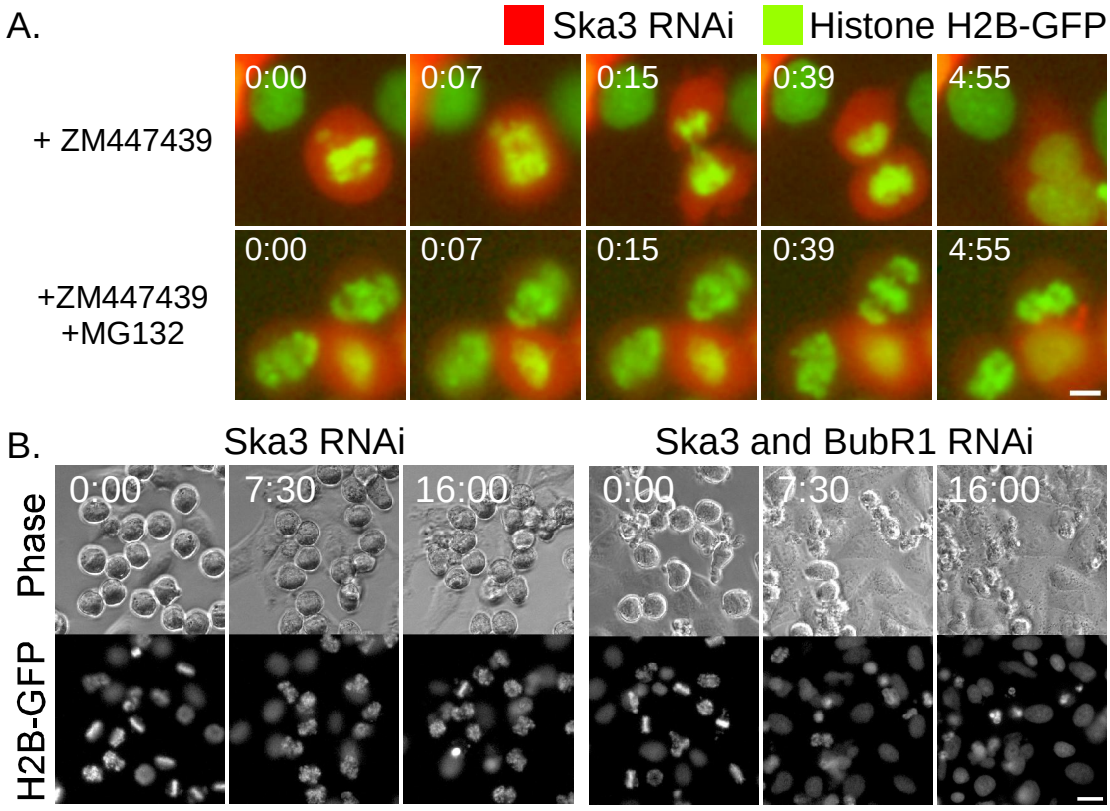
Supplemental Figure 1. Depletion of Ska3 by siRNA Induces Mitotic Arrest and Perturbs Chromosome Alignment at Metaphase.
(A) Hela cell cultures treated with a smartpool containing four siRNA duplexes to Ska3 induces mitotic arrest in a high proportion of cells. (B) Hela cells depleted of Ska3 by RNAi frequently fail to maintain alignment of chromosomes at the metaphase plate. A minority of paired chromosomes escape from the spindle midzone thus delaying metaphase alignment. Arrows denote chromosomes that have moved away from the metaphase plate. Control (Mock) cultures achieved metaphase alignment on average in 17.2 ± 3.9 minutes. In contrast Ska3 RNAi treated cultures achieved metaphase alignment in 33.6 ± 21.4 minutes. n = 50, Time = Minutes, Bar 5 μm.



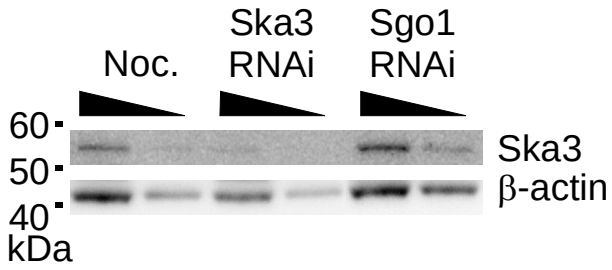
Supplemental Figure 2. Endogenous Ska3 Concentrates at the Outer Kinetochore Region in Prometaphase by Immunofluorescence Labeling.
(A) Immunolabeling of mitotic Hela cells labeled with anti-Ska3 antibody shows diffuse labeling in prophase with some concentration on the centrosomes. At prometaphase labeling is concentrated on kinetochores with some labeling of spindle fibers. Kinetochore labeling is diminished in anaphase and undetectable in telophase. Preimmune serum from the rabbit that was the source of the anti-Ska3 antibody shows no kinetochore labeling. Bar, 5 μm. (B) Comparison of intensity traces of Hela cells labeled with human anti-kinetochore serum or Hela cells expression Nuf2-GFP with anti-Ska3 labeling shows that Ska3 lies distal to the peak of anti-kinetochore labeling and largely coincident with Nuf2, indicating that Ska3 is concentrated in the outer kinetochore region. Bar, 1 μm.



Supplemental Figure 3. The Checkpoint Protein BubR1 Increases on Kinetochores After Chromosome Scattering. Control cells and cells treated with Ska3 siRNA were fixed and labeled with anti-kinetochore serum and anti-Bub1 antibody. Ska3-dpeleted cells with scattered chromosomes show a modest increase in kinetochore concentration of BubR1. In the graph, the black horizontal bars indicate averages; the colored boxes indicate standard errors, and the thin vertical "whiskers" indicate ranges.



Supplemental Figure 4. Mitotic Arrest Induced by Ska3 Depletion Is Abrogated by Loss of the Spindle Checkpoint.
(A) Selected frames from a video record of Hela H2B-GFP cells transfected with shRNA plasmid targeting Ska3. At 30 hours after transfection, cells were treated with the Aurora B inhibitor, ZM447439, or with both ZM447439 and the proteasome inhibitor MG132. ZM447439 alone inactivated the spindle checkpoint causing aberrant mitotic exit, flattening of the cells and reformation of the interphase nuclei. In cells co-treated with proteasome inhibitor, MG132, mitotic exit is blocked and chromosomes remain condensed. (B) Selected fluorescence and phase contrast frames from a video record of Hela H2B-GFP cells transfected with siRNA to Ska3 or doubly transfected with siRNA to Ska3 and to BubR1. Cells depleted of Ska3 alone arrest in mitosis. Doubly transfected cells undergo abnormal mitotic exit and flatten onto the substratum. Bars, 5 μm.



Supplemental Figure 5. Ska3 Protein Levels Remain High in Sgo1-depleted Cells.

Cells were arrested in mitosis by Nocodazole treatment, by Ska3 siRNA, or by Sgo1 siRNA. The mitotic arrested cells were collected by wash off and used to prepare cell extracts that were blotted for Ska3 and β-actin. Two lanes of each sample are shown, the second lane of each set was loaded at 50%. Ska3 siRNA but not Sgo1 siRNA causes a reduction in Ska3 protein level.

Supplemental Video Legends

Supplemental Video 1. Screening Video Showing that Cells Depleted of Ska3 Exhibit Altered Mitotic Progression.
Live cell time-lapse fluorescence images of a Hela H2B-GFP cell transfected with shRNAi plasmid targeting Ska3 (red) located above a non-transfected cell. The transfected cell delays at metaphase prior to arresting in mitosis with scattered chromosomes. The non-transfected cell proceeds normally through mitosis. Time is (hours:minutes) Bar, 5 μm.

Supplemental Video 2. siRNA to Ska3 Induces Metaphase Arrest Followed by Scattering of Chromosomes.
Time lapse phase and fluorescence images of Hela H2B-GFP cells transfected with siRNA targeting Ska3 are shown. Cells enter mitosis and align their chromosomes and delay at metaphase. After the extended metaphase delay, chromosomes scatter along the mitotic spindle. Frequently the spindles rotate within the cells. Time is (hours:minutes) Bar, 10 μm.

Supplemental Video 3. Co-depletion of BubR1 Alleviates the Mitotic Arrest Induced by Ska3 Depletion.
Time-lapse phase and fluorescence images of Hela H2B-GFP cells transfected with siRNA targeting Ska3 (upper video) or with a combination of siRNA targeting both Ska3 and BubR1 (lower video) are shown. Depletion of Ska3 alone induces mitotic arrest. Depletion of BubR1 overcomes mitotic arrest causing aberrant mitotic exit and cytokinesis. In some instances cells die after BubR1 depletion induced mitotic exit. Bar, 20 μm.