Supplemental Data

SAK/PLK4 Is Required for Centriole Duplication and Flagella Development

M. Bettencourt-Dias, A. Rodrigues-Martins, L. Carpenter, M. Riparbelli, L. Lehmann, M.K. Gatt, N. Carmo, F. Balloux, G. Callaini, and D.M. Glover

Supplemental Experimental Procedures

Flies and Husbandry

One *SAK* mutant allele, PBac{PB}SAKc06612 [S1], was employed in this study. It was acquired from Bloomington. The deficiency chromosome Df(3L)Pc-2q, ry[506] [078C05-06;078E03-079A01] that uncovers the region of the SAK gene (78D4) was kindly provided by A. Carpenter [S2]. We confirmed the mapping of c06612 as a single insertion [S1] by inverse PCR. This mutant is a hypomorph. All analysis was done on hemizygous flies, and thus we refer to those flies as *SAK* mutants in the text. GFP-PACT [S3] flies were kindly provided by Jordan Raff. OreR stocks were used as the wild-type. All flies were reared according to standard procedures and maintained at 25ºC.

Transfection and Treatment of Human Cells

HeLa and U2OS cells were kept in Dulbecco's modified Eagle's medium (DMEM; Sigma) containing 10% FCS (Gibco) at 37ºC and 5% CO_2 according to standard tissue-culture techniques. HeLa cells expressing GFP-centrin and kindly provided by Michel Bornens were kept as in [S4]. HeLa cells were seeded at 1.6×10^6 cells/well (6-well plate) 24 hr prior to transfection. Four microliters of 10 μM of each siRNA stock (×2) were mixed with 800 ul DMEM (w/o antibiotics) and 9 μl Transfast (TF, Promega) and allowed to complex for 15 min. Cells were washed with DMEM w/o antibiotics. The transfection mixture was added to the cells for 1 hr. Three milliliters of complete media (w/o antibiotics) were then added to the wells. Cells were split 4 hr afterward and diluted 1:4 in complete media. They were retransfected 20 hr later according to the same protocol (using half the amount of complexes). Analysis was performed at 48 or 72 hr after the first transfection. For inactivation of HsSAK, two double-stranded RNA oligonucleotides SAKa (from Ambion) and SAKb (from MWG) were ordered (see sequences in Table S1). A random sequence was used as a negative control-RSC (MWG). We obtained the best reduction in HsSAKmRNA when using a mixture of both oligos (see Figure S3). Treatment with both isolated SAKa and SAKb oligos led also to reduction in centrosome numbers in HeLa cells (data not shown; centrosome number assayed by γ-tubulin staining). Transfection of U2OS cells was performed as in [S5]. Twenty-four hours after transfection, AF (final concentration 1.6 ng/μl) or HU (final concentration 4 mM) was added to the cells. Cells were stained and analyzed 72 hr afterward.

RNAi in Drosophila *Cells and Making Stable Cell Lines*

dsRNA against SAK and GFP was made from genomic and plasmid DNA and transfected into *Drosophila* S2 [6, 7]. A list of primer pairs can be found in Table S1. *Drosophila* S2 cells were cultured and transfected with 40 μg of dsRNA and 20 μl of Transfast (Promega) in 6-well plates. Cells were harvested after 4 days unless otherwise indicated. To make stable cell lines of both myc-SAK and SAK-myc, we used the following protocol: 3 million cells were plated per well (6-well plate); the following day, 5 μg of the plasmid DNA construct of interest were mixed with 0.5 μg plasmid DNA-picoblast vector carrying blasticydin resistance (Invitrogen) in 100 μl of Serum Free Media (SFM); and 15 μl of Cellfectin (Invitrogen) were mixed with 100 μl of SFM. The two solutions were combined and incubated at RT for 30 min; 800 μl of SFM was then added to the mixture. Cells were then washed with SFM, and the transfection solution was added and left O/N. On the next day, cells were washed and fresh media was added. Two days afterward, new media containing 30 μg/ml of blasticydin was added to the cells. This was changed to 50 μg/ml of blasticydin 7 days afterward, and cells were kept on that concentration of antibiotics for 5 days. The cell line was then kept routinely at a concentration of 20 μg/ml blasticydin.

Constructs and Primers

Myc-SAK and SAK-myc constructs were made with the gateway system and vectors from Invitrogen and the DRGC (http://dgrc.cgb.indiana.edu/; see primers in Table S1). SAK cDNA (RE70136) was purchased from MRCgeneservice. Mutations in SAK were made in the SAK entry vector with the quick-change XL Site-Directed Mutagenesis Kit from Stratagene.

Phase and Fluorescence Microscopy

S2 cells were harvested and plated on glass coverslips and fixed 1 hr later in 4% formaldehyde in PHEM buffer (60 mM Pipes, 25 mM Hepes, 10 mM EGTA, 4 mM MgCl2). Cells were permeabilized, stained, and counted as in [S6]. Testes from pharate adults were prepared for staining with standard methanol and acetone fixation [S8]. For live images of testes preparations by phase microscopy, testes were dissected in 0.7% NaCl. HeLa and U2OS cells were fixed in ice-cold methanol for 4 min. They were permeabilized and stained as described previously [S6]. For γ-tubulin, D-PLP, and α-tubulin staining, larval brains were prepared and treated as in [S9]. Brain squashes were carried out as previously [S10]. After being frozen in liquid nitrogen, slides were fixed in ice-cold methanol for 10 min. They were then rehydrated in PBS + 0.1% Triton for 10 min; immunostainning was then performed for phospho-histone H3 and DAPI [S6]. The mitotic index was determined as the ratio of phospho-histone-H3-positive (mitotic) cells per total (DAPI-stained) cells. Phase-contrast photographs were taken with a Nikon Coolpix 990 digital camera on a Nikon Microphot-FX microscope. An axiovert 200M microscope was used for observation and countings of tissue-culture cells and brain squashes. Images on this microscope were acquired with a Photometrics Cool SNAP HQ camera. Images of imunostained testis were acquired with a confocal scanning head (model 1024; Bio-Rad Laboratories) mounted on an Optiphot microscope (Nikon). The figures shown are the maximum-intensity projections of optical sections acquired at 0.2–0.5 μm steps. All images were prepared for publication with Adobe Photoshop.

Correlative Immunofluorescence and Electron Microscopy Analysis of Mitotic Cells and Testes

S2 cells in coverslips were immersed in 4% paraformaldehyde (EM grade, methanol free) in PBS for 15 min and then immunostained for centrin1 and α-tubulin. Fields containing 20–30 cells were selected under a Zeiss Axioskop 40 epifluorescence microscope equipped with a 40× objective and imaged with a cooled CCD camera (AxioCam HRm rev. 2, Zeiss). Selected fields were then marked under a 20× objective by scraping extra cells from the edges with a fine needle. Coverslip cultures containing selected fields were immersed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) for 2 hr at 4ºC. After they were washed three times for 30 min in phosphate buffer, the cells were postfixed with 1% OsO4 for 1 hr at 4ºC, washed once in buffer, and then in distilled water. Cells were then dehydrated in a graded series of ethanols and flat-embedded in a mixture of Epon and Araldite. After polymerization for 2 days at 60ºC, the coverslips were removed from the resin after a short immersion in liquid nitrogen. Ultrathin serial sections were obtained with a LKB ultratome, stained with uranyl acetate and lead citrate, and observed and photographed with a Philips CM10 electron microscope at 80 kV. Only those cells in which it was possible to obtain several consecutive sections through their equator or their poles were recorded. Testes were dissected in phosphate buffer and fixed, postfixed, and washed as above, but after the distilled water wash, the samples were stained 1 hr in block in uranyl acetate. They were washed again in distilled water, dehydrated in a series of ethanols, and embedded in Epon-Araldite. Sectioning and staining was as above.

Antibodies

We used the following antibodies: rat anti-α-tubulin-YL1/2 (Oxford Biosciences; 1/50); mouse anti-γ-tubulin-GTU88 (SIGMA; 1/100); rabbit anti-centrosomin (Cnn, produced in our own lab rb7647; 1/300); rabbit anti-CP190 (Rb188 [S11]; 1/300); mouse anti-myc-9E10 (abcam; 1/1000); rabbit anti-phosphohistone H3 (upstate; 1/500); anti-human-centrin 1 [S12] (1:400); actin (SIGMA, 1/2000); rabbit anti-PLP was kindly provided by Jordan Raff [S3] (1/1500); mouse anti-Centrin-2 antibody was kindly provided by Jeff Salisbury (hCetn2.4 [S13]; 1/1000); and mouse anti-polyglutamylated tubulin antibody (GT335 [S14]; 1/1500) was kindly provided by Carsten Jenke. For antibody production, a recombinant protein of the C terminus of SAK was expressed in *E. coli* with pET23b vector and injected into rabbits (Harlan Sera, UK).The secondary antibodies used (1/100) to detect all antigens were conjugated with Rhodamine Redex, Peroxidase (Jackson Immunochemicals), FITC (SIGMA), or Alexa 350 (Molecular Probes) and had minimal cross reactivity to other species.

DNA and Annexin V Profiling by FACS Analysis

Cells were harvested by trypsinization, pelleted, and resuspended in 1 ml 70% ethanol (−20ºC) while vortexing and fixed for 1 hr (or o/n) at −20ºC. Cells were then washed in PBS + 0.1% Triton and resuspended in 0.5 ml PBS (36 μg/ml RNase A) and (120 μg/ml propidium iodide) and incubated for 30 min at 37ºC in the dark. Data from 30,000 cells was acquired on FL2 on a Becton Dickinson FACScan or a Becton Dickinson LSR. Results were analyzed with Summit from Dako Cytommation. Annexin V profiling was performed with the Annexin-V-FLUOS Staining Kit (Roche) according to manufacturer's instructions.

Automated Analysis of Mitotic Index

Cells were fixed in a 96-well plate with 4% formaline in 1× PBS for 15 min at RT. Cells were then processed for immunostaining as above with rabbit anti-phospho histone H3 (Upstate 06-570; 1:500) and counterstained with DAPI (1/100) in PBS for 30 min in the dark. Cells were washed and analyzed on Zeiss Axiovert 200M for DAPI (wavelength 1) and 594 (wavelength 2) with Metamorph software. Each siRNA treatment was replicated in six wells, analyzed 16 times at various points in the well. Journals were created in Metamorph, to automatically count positive cells from w1/w2, and data are expressed as % of phospho-histone-H3-positive cells relative to total (DAPI) cells counted.

QRT-PCR

RNA was extracted from cells with the Qiagen Rneasy Protect Mini Kit (Quiagen) or Trizol (Invitrogen) and prepared according to manufacturers' instructions. RNA was standardized to 50–200 ng/μl after 260/280 analysis. cDNA was synthesized with the Ambion Cells-to-cDNA kit according to the manufacturer's instructions. cDNA QRT was performed with Sybr Green PCR Mastermix from ABI with a ABI Prism 7000 machine. Levels of gene expression are shown as a ratio of Ribosomal RNA (Human cells) or eIF4α (*Drosophila*) in the reaction, then normalized to GFP, RSC, or wild-type (WT) control.

Mathematical Modeling and Counts

The centriole-duplication model can be found in Appendix S1. Mitotic indexes and mitotic centrosome defects after RNAi (Figure 1) were counted after staining of cells for γ-tubulin, α-tubulin, and DNA (DAPI) [S6]. The same staining was performed in larval brains for counts of mitotic centrosome defects with Toto-3 (Molecular Probes) as a DNA counterstain. Mitotic indices in larval brains were counted in preparations of squashed larval brains stained with phospho-histone H3 (to label mitotic cells) and DAPI (to label nuclei and chromosomes). A minimum of 2000 cells were counted in each independent experiment. Counts were performed blindly. Interphase centrosome counts in *Drosophila* tissue-culture cells (Figure 2) were performed after staining for D-PLP, α-tubulin, and γ-tubulin. A minimum of 150 cells were scored for the number of PLP dots per cell in each independent experiment. The number of centrioles in cells post-MII was counted by scoring preparations of testes from WT (w; GFP-PACT; +) and *SAK* mutant [w; GFP-PACT; SAK^{c06612}/Df(3L)Pc-2q] flies, stained for α-tubulin and DNA (Figure 4). A minimum of 150 cells were scored by pooling results from four different experiments. Only cells with well-defined cellular shape were counted. *SAK* mutant spermatocytes that lack centrioles have irregular nuclear size and number and cell shapes vary, so they can be difficult to identify. This could have potentially led to an underestimation of the percentage of cells with no centrioles. Images of each 16-cell cyst were initially taken at low magnification (25× objective) with maximum-intensity projections of optical sections acquired at 0.2–0.5 μm steps. More-detailed images of cells were then acquired with a 100× objective. Images were then analyzed with digital zoom to correctly identify the number of centrioles in each centrosome. Centriole number in human cells was scored with the centrin-2 antibody for HeLa cells and the GT335 antibody for U2OS cells, together with α-tubulin and DNA (Figure 5). A minimum of 100 interphase cells were scored for the number of centrioles per cell in each condition in each experiment. A minimum of 80 mitotic cells were scored for mitotic abnormalities in HeLa cells in each experiment (Figure 5).

Supplemental References

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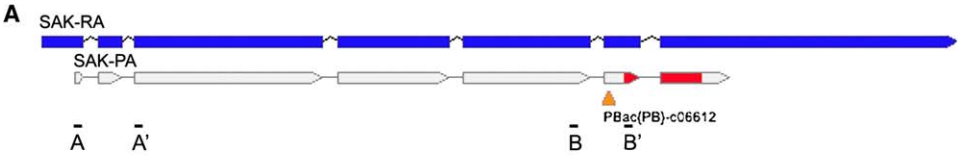

B

$SAK^{c06612}/TM6B$ X $Df(3L)Pc\text{-}2q/TM6B$

Genotype analysed	$SAK^{c06612}/Df(3L)Pc\text{-}2q$						
Stage (number of flies of selected genotype reaching that stage)	Pupae (n=809)	Adults (pupae not selected n=72)		Adults (pupae selected and isolated n=112)		Fertility (selected and crossed with OreR)	
Sex	Both	M	F	M	F	M (n=25)	F (n=5)
Results Observed (expected-100%)	96% *	33%	5%	79%	51%	0%	0%

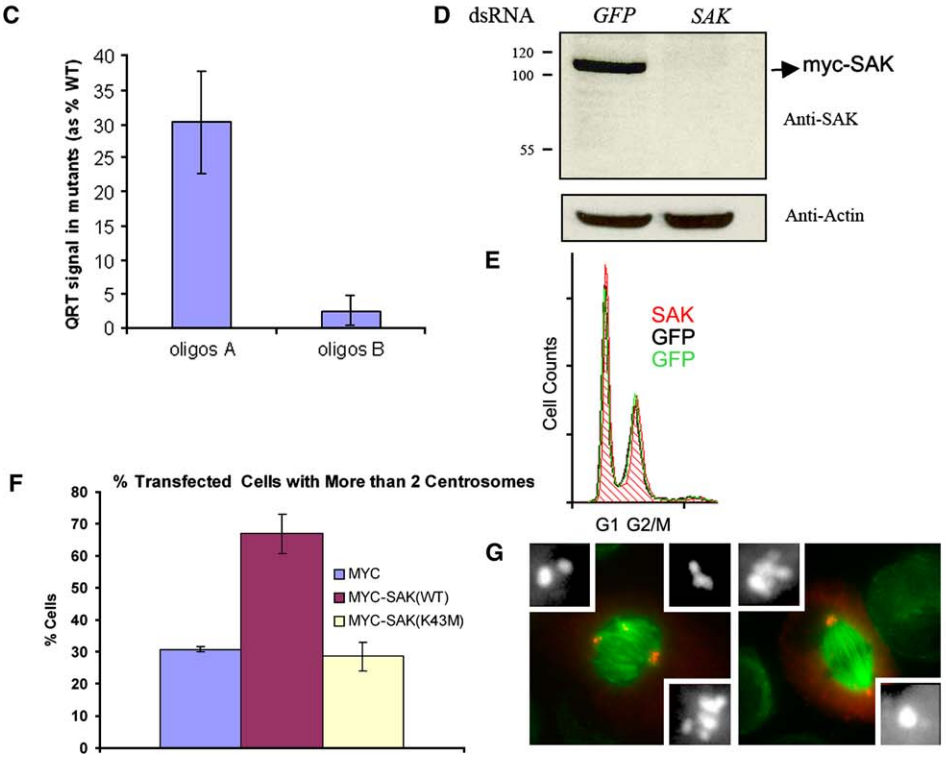

Figure S1. *SAK* Mutant Flies Are Semilethal and Sterile

(A) Schematic representation of the SAK transcript (SAK-RA) and SAK open reading frame (SAK-PA) as assigned in FlyBase. The red region corresponds to the polo-box domain. The orange triangle marks the site of insertion of the transposon element PBc06612 as assigned originally by Excelexis [S1] and confirmed by us by inverse PCR.

(A and A′) and (B and B′) indicate oligos used in QRT-PCR.

(B) All experiments performed in this paper were done on the hypomorph $SAK^{c06612}/Df(3L)Pc\text{-}2q$. *Df(3L)Pc-2q* is a chromosome carrying a deficiency that uncovers the SAK gene. This is the strongest allelic combination we have, and it avoids the possibility of erroneous phenotypes resulting from second-site mutations on the mutant chromosome.

* Although the majority of $SAK^{c06612}/Df(3L)Pc\text{-}2q$ flies pupate, they are delayed by approximately 1 day as compared to their siblings of genotype SAK^{c06612}/TM6B or *Df(3L)Pc-2q*/TM6B. Even though all mutants eclose (all pupae cases are found empty), they die shortly after eclosion. This is more pronounced in females. When mutant pupae were removed from their original vial and put in a separate vial with a small piece of paper on top of the food (to avoid flies getting stuck in the food) the percentage of survival was much higher (column corresponding to "pupae selected and isolated"). Many of these flies were uncoordinated and died soon after eclosion. Mutant flies that survived without isolation were crossed with OreR (WT) flies to test for fertility. Both males and females were sterile. SAK^{c06612} homozygotes are also sterile (n = 10 males and 10 females), but they have higher viability relative to $SAK^{c06612}/Df(3L)Pc\text{-}2q$ (75% of the flies that pupate eclode and survive, n = 1091). We have also observed centrosome defects in mitotic cells of brains of homozygous larvae, but less pronounced than the ones described in hemizygous flies.

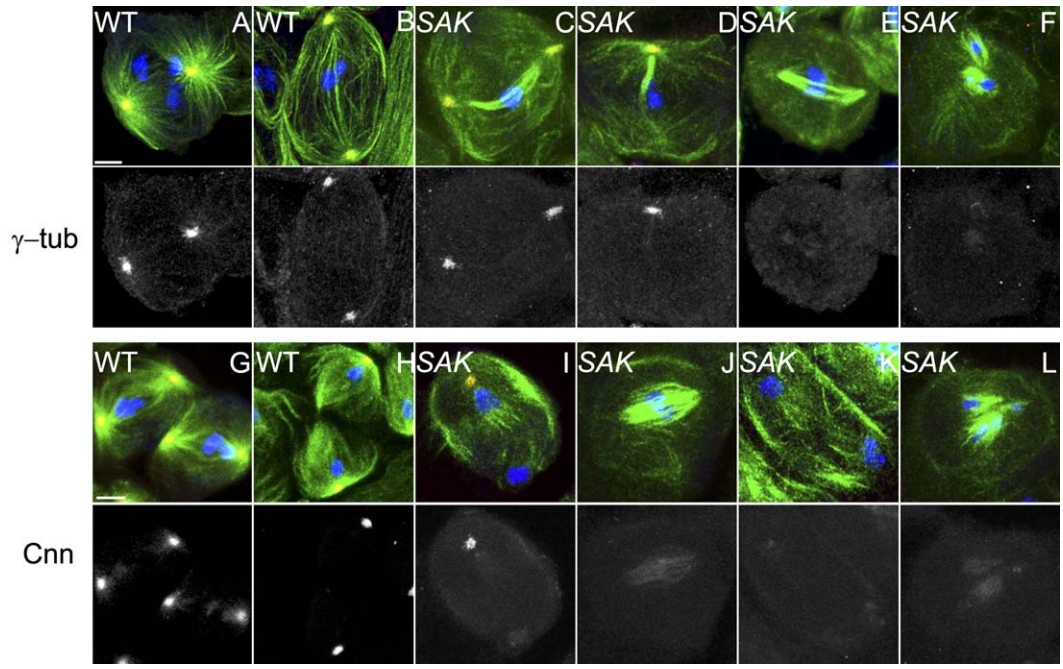

Figure S2. SAK Is Important for Centrosome Integrity in *Drosophila* Testes

Wild-type (WT) and *SAK* mutant primary spermatocytes in meiosis were stained for α-tubulin (green), DNA (blue), and γ-tubulin (red; top panel and also lower monochrome image) or Cnn (red; lower panel and also lower monochrome image). The majority of the mutant cells do not show centrosome markers at both poles, but cells with two (C) or just one centrosome (D and I) can also be seen. Whereas some cells developed very abnormal spindles (F and L), others were able to make bipolar spindles (E, I, J, and K) and segregate their DNA (K). In many acentrosomal spindles, microtubules appeared to assemble outward from the chromosomes (F, J, and L). The scale bar represents 5 μm.

(C) QRT-PCR for SAK. Because the abundance of SAK protein is very low, we used QRT-PCR to analyze whether the insertion c06612 could lead to a truncated mRNA. Oligos used are indicated in (A). Data were first normalized against levels of eIF4α. Signal for mutants (SAK^{c06612}/*Df(3L)Pc-2q*) is shown as a percentage from WT. Note that RNA for SAK is being transcribed (amplification from oligos A) from the chromosome carrying c06612, but it is truncated (only background amplification with oligos (B), due to the presence of the transposon. This may lead to production of a truncated protein, which may have some residual function and hence explain the hypomorphic nature of the allele. Note that levels of expression may be lower that expected from a hemizygous fly (50%). Thirty brains of each genotype were used in each experiment.

(D) RNAi against SAK reduces the amount of myc-SAK protein in an S2 cell line stably expressing that protein from the Act5C promoter. Endogenous SAK protein is not visible in a whole-cell lysate western (predicted MW for SAK-86 kDa) when several antibodies we have generated against different parts of the protein were used (not shown). The same blot was reprobed for actin as a loading control.

(E) No changes in FACS profile of DNA content were observed 4 days after RNAi for SAK as described before [S6].

(F) Expression of myc-SAK, but not myc or myc-SAKK43M (mutation in the nucleotide binding site), leads to an increase in D-PLP-foci. Cells were transfected with those constructs, they were fixed 2 days later, and the number of D-PLP foci was counted in myc-positive cells.

(G) myc-SAK-induced foci accumulated at the poles of multipolar and bipolar spindles in mitosis. Green shows α tubulin, and red shows myc-SAK. The insets show 4× myc-SAK.

Error bars represent standard error of the mean (SEM).

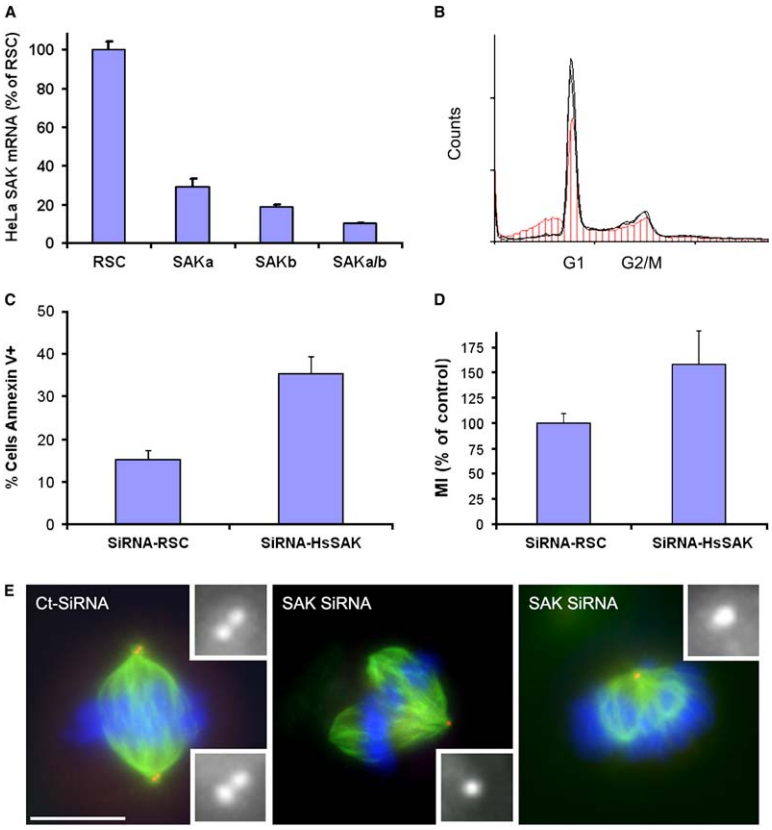

Figure S3. Depletion of HsSAK Leads to Cell Death, Higher Mitotic Index, and Mitotic Defects

(A) Transfection of HeLa cells with SiRNA for SAK leads to depletion of more than 70% of its RNA as measured by QRT-PCR. SAKa and SAKb are two different siRNAs; SAKa,b is a combination of both in the same treatment. RSC denotes random sequence control. The combination of oligos SAKa and SAKb was chosen for further experiments because it gave the best depletion of SAK's message.

(B) siRNA for SAK leads to an increase in the subG1 population as seen by analysis of propidium-iodide content by FACS. Black profile shows control cells, and red profile shows cells treated with siRNA for SAK. Note the 3–4-fold increase in the percentage of apoptotic cells (subG1 population) after depletion of SAK.

(C) Treatment of cells with siRNA for SAK leads to a significant increase in early apoptotic cells as measured by the ability of annexin V to bind externalized phosphatidyl serine in those cells.

(D) Treatment of cells with SiRNA for SAK leads to an increase in mitotic index as measured by automatic counting of nuclei (DAPI) and mitotic cells (phospho-histone H3). Data is shown as average; error bars are SEM.

(E) Mitotic abnormalities following SiRNA of SAK in U2OS cells. Red shows GT335, green shows α-tubulin, and blue shows DNA. Insets show GT335-magnification 4×.

Table S1. List of Primers Used

	Forward	Reverse
Drosophila SAK QRT	CAATCGGGCGTTTGGAGAAAC	AACGCGGTTAGTGAGTCCAGTGCC
Drosophila eIF4α QRT	ATGAACTTGCGCGAGGAGTTGC	GGATAGCAATCGAGAAGGTGGCAGT
Drosophila SAK Gateway System-C-terminus fusion	GGGGACAAGTTTGTACAAAAAAGCAGGCT TCATGTTATCCAATCGGGCGTTTGGA	GGGGACCACTTTGTACAAGAAAGCTGGG TCAAGAAGCATGCGATTATAATA
Drosophila SAK Gateway System-N-terminus fusion	GGGGACAAGTTTGTACAAAAAAGCAGGCTTC ATGTTATCCAATCGGGCGTTTGGA	GGGGACCACTTTGTACAAGAAAGCTGGGT CTTAAAGAAGCATGCGATTATAATA
Drosophila SAK-T7 RNAi primers	TAATACGACTCACTATAGGGAGAATACGG GAGGAATTTAAGCAAGTC	TAATACGACTCACTATAGGGAGATTATAAC GCGTCGGAAGCAGTCT
Drosophila SAK-T7 RNAi primers	TAATACGACTCACTATAGGGAGACGCT ATATGAACCACATCGCCAGAC	TAATACGACTCACTATAGGGAGAAACATAA AGGGATGGCAGAGAACAG
Drosophila GFP-T7 RNAi primers	TAATACGACTCACTATAGGGA GACTTCAGCCGCTACCCC	TAATACGACTCACTATAGGGAGATGTCG GGCAGCACG
Drosophila polo-T7 RNAi primers	TAATACGACTCACTATAGGGAGACGTTCT CCGCTTTGTGCTTGGTTTTCGTG	TAATACGACTCACTATAGGGAGACGCTTGT AGGTTTTCCGCTGGTTGATGTCG
Drosophila polo-T7 RNAi primers	TAATACGACTCACTATAGGGAGAGGAGTT CGAATGCCGCTACTACATT	TAATACGACTCACTATAGGGAGATCAG ACAAGAGCTGGGCAAGAACAT
SAKa SiRNA	GGUGGAAAUACAUUGCCAA(dTdT)	
SAKb SiRNA	GGACCUUAUUCACCAGUUA(dTdT)	
RSC SiRNA	GCUAUGUGACGUAGAGCGA(dTdT)	
HS-SAK QRT-PCR	AGGATCATTTGCTGGTGTCTACAG	GAAGGATGTTTCAATTGGCAATGTATTTTC
SAK-K43M	CCAGGATGTGGCCATAATGATGAT CGATAAAAAAC	GTTTTTTATCGATCATCATTATGGCCACAT CCTGG
SAK-1050-antibody	ATCGGAATTCGCAGAGCCAAATTGGCCAGGA	CAGTGAATTCAAGAAGCATGCGATTATAATA