Supplemental Data

SAK/PLK4 Is Required for Centriole Duplication and Flagella Development


Supplemental Experimental Procedures

Files 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)Po-2q, y[80E] [078C05;06;078E03;079A01] that uncovered the region of the SAK gene (7F04) 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. OeR 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% CO₂ 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 × 10⁵ cells/well (6-well plate) 24 hr prior to transfection. Four microliters of 10 μM of each siRNA stock (≥2) were mixed with 800 μl 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 HisSAK, double-stranded RNA oligonucleotides SAKa (from Ambion) and SAKb (from MWG) were ordered (see sequences in Table S1). A random stranded RNA oligonucleotides SAKa (from Ambion) and SAKb (see Figure S3). Treatment with both isolated SAKa and SAKb resulted in the best reduction in HsSAK mRNA when using a mixture of both oligonucleotides. 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. OeR stocks were used as the wild-type. All flies were reared according to standard procedures and maintained at 25ºC.

Analysis of Mitotic Cells and Testes

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 before, 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-CPI90 (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 Immunochromicals), 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 (−20°C) while vortexing and fixed for 1 hr (or room temperature) at −20°C. 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 37°C in the dark. Data from 30,000 cells was acquired on FL2 on a Becton Dickinson FACScan. Results were analyzed with Summit version 3.1. Data was 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 using the Annexin-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

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 PBe06612 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 SAKc06612/Df(3L)Pc-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 SAKc06612/Df(3L)Pc-2q flies pupate, they are delayed by approximately 1 day as compared to their siblings of genotype SAKc06612/TM6B or Df(3L)Pc-2q/TM6B. Even though all mutants eclosed (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. SAKc06612 homozygotes are also sterile (n = 10 males and 10 females), but they have higher viability relative to SAKc06612/Df(3L)Pc-2q (75% of the flies that pupate eclosed 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 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 PBe06612 as assigned originally by Excelexis [S1] and confirmed by us by inverse PCR.

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(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 eIF4a. Signal for mutants (SAKc06612/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 than 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 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.
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×.
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<td><strong>Forward</strong></td>
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