Supplemental Data

EXPERIMENTAL PROCEDURES

Drosophila stocks

Sevelin strain was used as wild type. *dwee1*^{ES1} and *Df(2L)dwee1*^{W05} [1], *klp61f*^{3} [2, 3] and *myc-klp61f*^{WT} [2] have been described before. Homozygotes were identified by their lack of balancer markers (Cy, GFP or Tb). *myc-klp61f*^{WT} and *myc-klp61f*^{3YF} transgenic chromosomes are balanced over CyO. *klp61f*^{3} mutants that carry the *myc-klp61f* transgenes were constructed with standard Drosophila genetics.

Production of Antibodies Against dWee1

A 20 amino acid peptide spanning D41-R60 of dWee1 was used for antibody production: CDDFDKDTPEGASPQHPLQQR. Peptide synthesis and conjugation, immunosorbent production, and affinity purification for antibodies were done commercially (Bethyl Laboratories, Inc).

Immunoprecipitation and Western Blots

Immunoprecipitation and western blotting were performed as previously described [4], using 10 µl protein G beads (Pierce) and rat anti-KLP61F serum [5] at 1:250 dilution to precipitate KLP61F. The following antibodies were used in western blots: rat anti-KLP61F serum, 1:2500; rabbit anti-dWee1, 1:1000; mouse anti-phosphotyrosine, 1:500 (Millipore); mouse anti-His, 1:1000 (Amersham), rabbit anti-GST, 1:1000; HRP-conjugated anti-rat (Jackson ImmunoResearch), anti-mouse, and anti-rabbit secondary antibodies (Amersham), 1:5000.

Protein Expression

Generation of GST-dWee1 and His-KLP61F has been described previously [6, 7]. His-Head^{WT} and His-BimC were cloned using Gateway technology (Invitrogen) with KLP61F cDNA as a template. pDONR 221 vector was the entry vector and pDEST17 was the expression vector. KLP61F cDNA was cloned by PCR from a cDNA library from 0–4 h *Drosophila* embryos [8]. His-Head^{3YF} was generated using PCR site-directed mutagenesis to mutate Y23, Y152, and Y207 to phenylalanines within pDONR 221-Head^{WT} and then cloned into pDEST17 vector.

pDEST17 constructs were expressed from BL21-AI™ One Shot® Chemically Competent E. coli (Invitrogen). Cell lysates were purified over a Talon Metal Affinity Resin (Clontech).

Kinase Assays

Substrates were pre-incubated in 20 μl reaction buffer (50mM Hepes, 15mM MgCl2, 1mM EGTA, 10% glycerol, 10mM DTT, 0.1mM ATP with 2mM sodium vanadate, 10mM β-glycerolphosphate, 1mM sodium metabisulfite, 1mM benzamidine, 5ug/ul aprotinin, 5ug/ul leupeptin) for 30 min at 30^{o}C to saturate phosphorylation by possible co-purifying kinases, followed by the addition of equimolar GST-dWee1 and 10 μCi of [γ-^{32}P] ATP and further incubation at 30^{o}C for 30 min. Reactions were terminated by addition of SDS sample buffer, resolved by SDS-PAGE and transferred to PVDF membranes for detection by Phosphorimager (Molecular Dynamics). Membranes were western blotted with anti-phosphotyrosine antibodies.

Trypsin Digestion and Mass Spectrometry

All concentrations are final, unless otherwise noted. 10 μl kinase reactions were brought up to 25 μl with reduction solution (100 mM ammonium bicarbonate, 2M Urea). Proteins were reduced for 30 min at 60°C with DTT (2μM) and cooled to 22°C. Alkylation was performed with Iodoacetamide (10 μM) in the dark at 22°C for 45 min. Proteins were then digested with modified trypsin (1:50 w/w) at 37°C overnight with rocking. Resulting peptides were prepared for mass spectrometry as previously described [4].

Matrix-assisted laser desorption ionization time of flight (MALDI-TOF) mass spectrometry (Voyager-DE STR, Perkin Elmer) was used to obtain peptide mass information. Coverage of proteins was determined by peptide-mass fingerprinting with ProteinProspector (http://prospector.ucsf.edu/), searching against *Drosophila* entries in the National Center for Biotechnology (NCBI) nonredundant protein database. KLP61F phosphopeptides were identified by the following criteria: (1) showed a mass increase of 80Da after incubation with GST-dWee1; (2) contained a single tyrosine; and (3) detected only in *in vitro* kinase reactions containing both GST-dWee1 and His-KLP61F.

Generation of Myc-KLP61F^{3YF} Transgenic Flies

KLP61F cDNA was released from Pwum2-KLP61F [2] by digestion with KpnI and EcoRI, gel purified and further digested with BsaI to remove a fragment that encodes the N-terminus including Y23, Y152, and Y207. A similar digest was performed on pDONR 221-Head^{3YF} to obtain the corresponding fragment. A Kpn1 site, 3 Myc tags, and a new start site was introduced into the KLP61F^{3YF} N-terminus encoding fragment by PCR. The resulting piece was ligated to the C-terminal fragment of KLP61F^{WT} cDNA. The resulting Myc-KLP61F^{3YF} cDNA was re-introduced into the Pwum2 transformation vector. Pwum2-KLP61F^{3YF} was verified by sequence analysis of the region encoding head and tail domains. Injections, generation of individual stable transformants, and balancing with balancer chromosome were services performed by Bestgene, Inc. Five independent transgenic lines with a single insertion in the second or third chromosome were obtained.

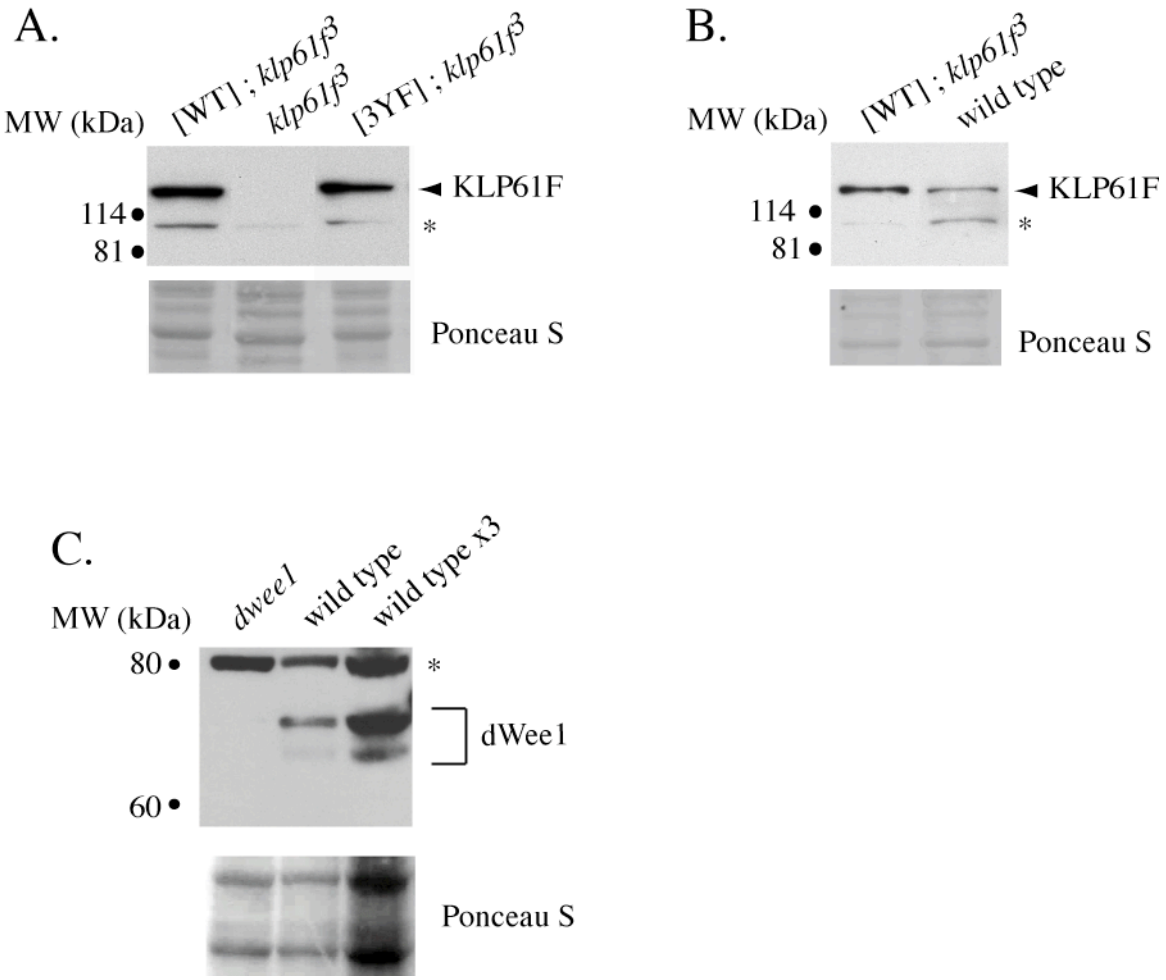
Cytological Analysis

Brain squashes were carried out as described before [6] using wandering third-instar larvae. 0-2 hr embryos were fixed and stained as previously described [4]. Antibodies used were: rabbit anti-CNN, 1:5000 (gift from Tim Megraw); rat anti-α-tubulin, 1:500 (Abcam Inc); and mouse anti-Myc, 1:1000 (clone 9B11, Cell Signaling). FITC- or rhodamine-conjugated secondary antibodies (Jackson Labs) were used at 1:500. DNA was stained with 10 μg/ml bisbenzimide (Molecular Probes). Samples were imaged on a Leica DMR microscope with a Sensicam CCD camera and Slidebook software (Intelligent Imagining Innovations). Cycle number for embryos was determined by nuclear density.

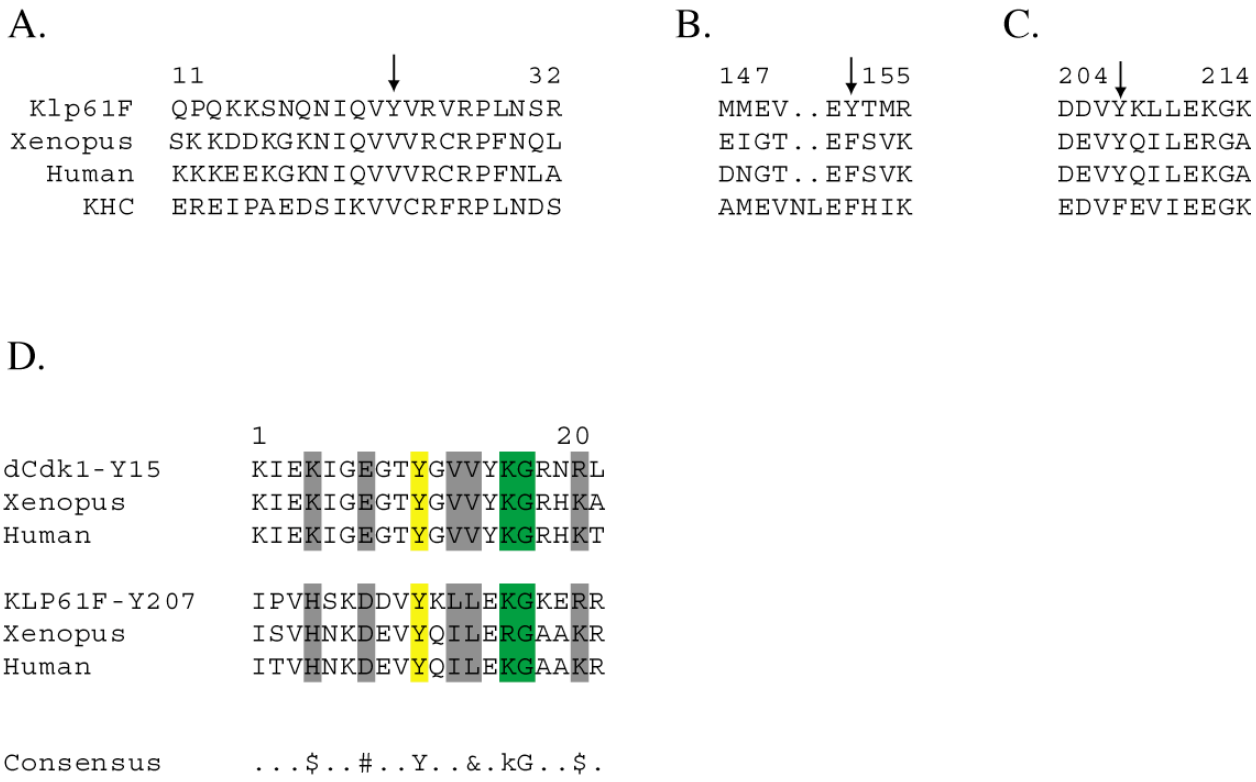
Statistical Analysis

P-values were calculated using a student t-test with 2-tailed distribution for percents of dead pupae and percents of aneuploid nuclei. P-values were calculated using exact binomial tests for percents of mitotic spindle defects.

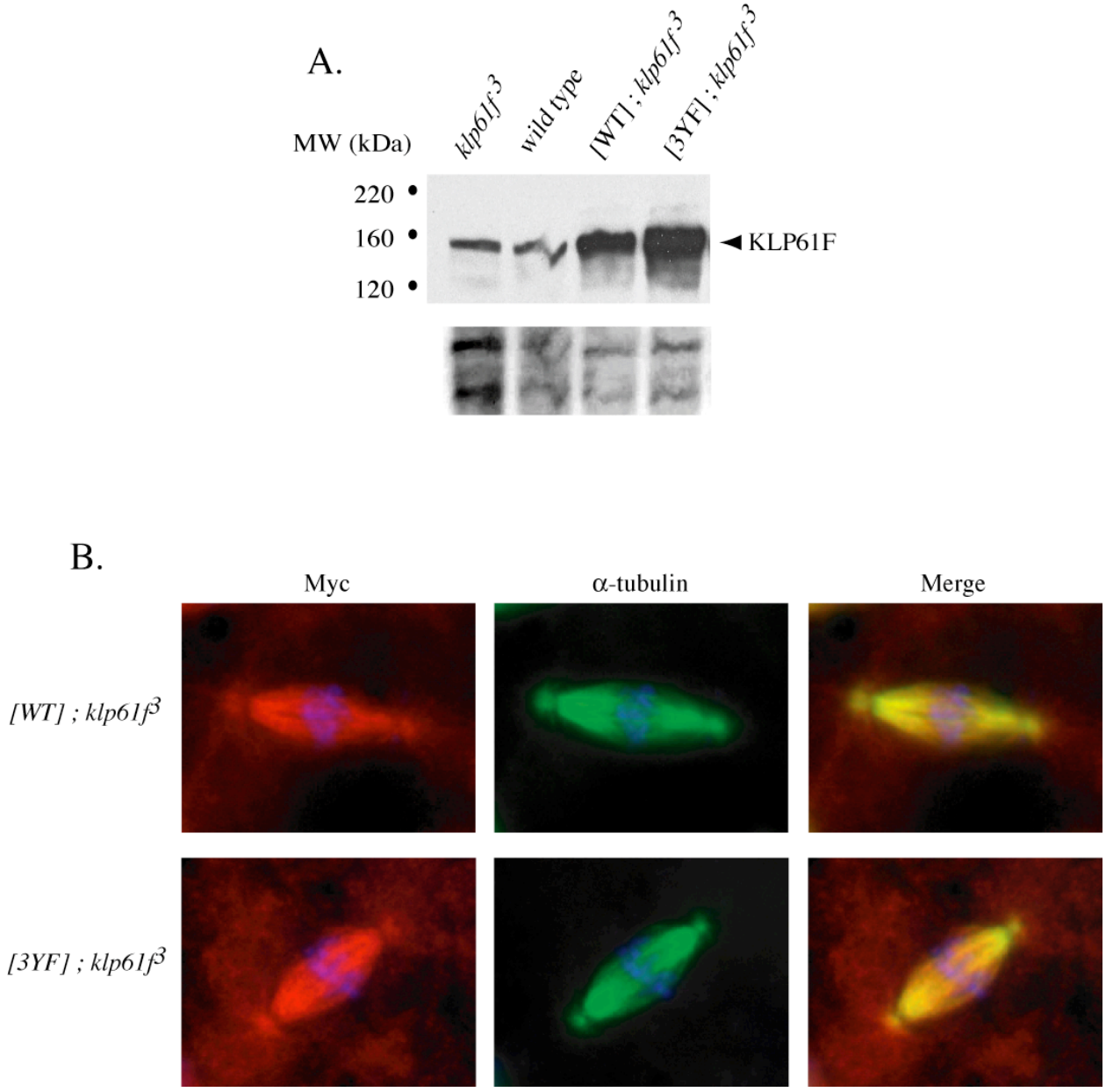
SUPPLEMENTAL FIGURES



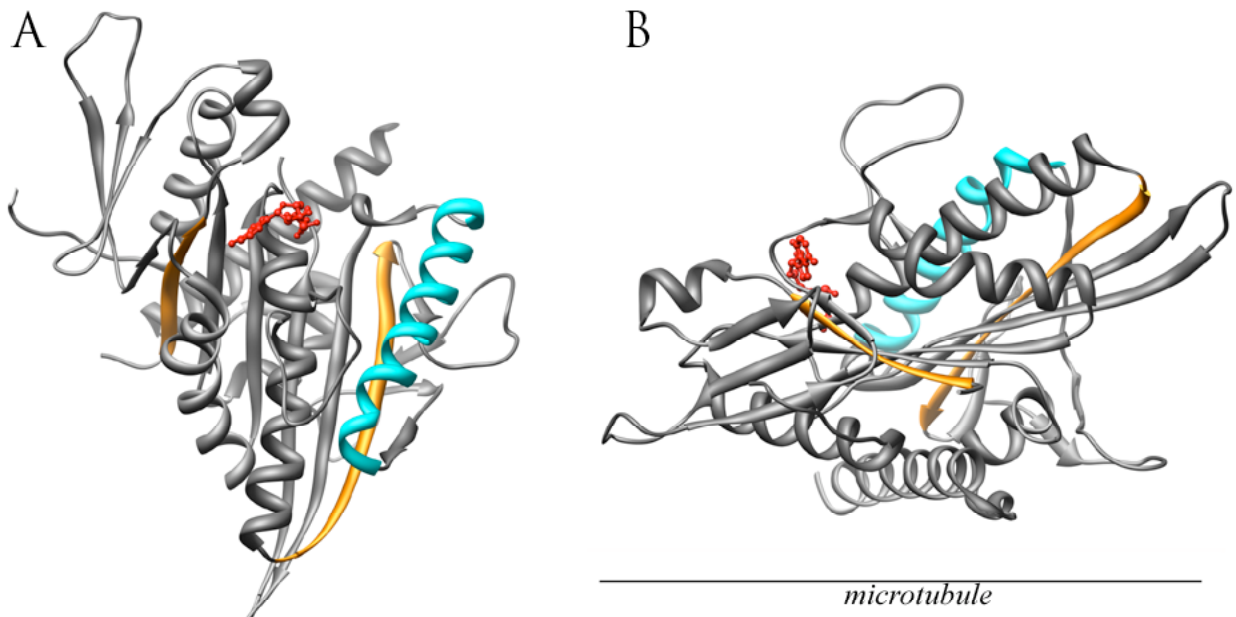
Supplemental Figure 1: Specificity of antibodies made against KLP61F or dWee1. **(A)** Extracts from *klp61f*^{3} homozygous mutant larvae expressing either a wild type KLP61F transgene ([WT]; *klp61f*^{3}), a KLP61F^{3YF} transgene ([3YF]; *klp61f*^{3}) or no transgene (*klp61f*^{3}) were Western blotted with an anti-KLP61F serum. Arrowhead marks the band at the predicted size for KLP61F (120kDa) that is absent in *klp61f*^{3} mutants but restored upon expression of KLP61F transgenes. **(B)** Extracts from *klp61f*^{3} homozygous mutant larvae expressing a wild-type KLP61F transgene and extracts from wild-type larvae were Western blotted with anti-KLP61F antibody. Arrowhead marks the band at the predicted size for KLP61F (120kDa) that is seen in wild type larvae **(C)** Extracts from *dwee1* mutant embryos (*dwee1*), syncytial wild-type embryos ('wild-type', equal loading and 'wild-type X3', triple loading) were Western blotted with affinity-purified polyclonal antibodies against a dWee1 N-terminal peptide. Bracket marks the two protein bands from wild type embryos that correspond to the predicated size of dWee1 (69kDa) but are absent in *dwee1* mutant embryos. * indicates apparently non-specific bands. Ponceau S staining indicates relative amounts loaded.



Supplemental Figure 2: Sequence alignment of KLP61F phospho-peptides and sites of Wee1 phosphorylation. Three peptides within the KLP61F head domain that were identified by MALDI-TOF mass spectrometry as possible phosphopeptides are depicted. Alignment with other metazoan Kinesin-5 motors and a Kinesin-1 motor, Kinesin Heavy Chain (KHC), are shown below the peptides. Each KLP61F peptide contains a single tyrosine (arrow), which is not conserved in the KHC head domain. **(A)** Y23, **(B)** Y152, **(C)** Y207. (**D**) Alignment of Y15 phosphoacceptor in metazoan Cdk1 homolgs (top) and Y207 in metazoan Kinesin 5 motors (bottom) reveal a possible Wee1 phosphorylation consensus motif. Tyrosines of interest are shaded yellow. Residues shaded gray indicate conserved similar amino acids. Residues shaded green indicate residues that are highly conserved (>83%). In the predicated consensus motif, $ = basic residue, # = acidic residue, & = branched residue, lower case = 83% (for k) conserved, upper case = 100% conserved.



Supplemental Figure 3: Myc-KLP61F^{3YF} transgene in syncytial embryos from *klp61f^{3}* heterozygous mothers. (A) Extracts from embryos laid by wild-type mothers, *klp61f^{3}* heterozygous mothers, and *klp61f^{3}* heterozygous mothers expressing the Myc-KLP61F^{WT} or Myc-KLP61F^{3YF} transgenes were resolved by SDS-PAGE and subjected to western blot analysis with anti-KLP61F serum (top panel). Ponceau S staining before western blot analysis indicates levels or protein loading (bottom panel). A greater amount of *klp61f^{3}* extracts than WT extracts were required to produce equivalent KLP61F signal, consistent with the former containing less KLP61F protein. **(B)** Embryos from heterozygous *klp61f^{3}* mothers carrying Myc-KLP61F^{WT} or Myc-KLP61F^{3YF} transgenes were fixed and stained with anti-Myc antibodies (red), anti-tubulin antibodies (green) and for DNA with Hoechst (blue). Representative images of metaphase spindles are shown.



Supplemental Figure 4. The regions of human Eg5 head/motor domain that are predicted to correspond to the regions of KLP61F with potential phospho-acceptor tyrosines. Y23 in KLP61F is predicted to fall within β-sheet 1 (shorter yellow sheet). Y152 is predicted to fall within β–sheet 4 (longer yellow sheet). Y207, the only one conserved in metazoan Kinesin-5 homologs, is predicted to fall within α-helix 3 (blue). View (A) illustrates the proximity of α-helix 3 to the bound nucleotide (red). Switch I region that is involved in nucleotide sensing occurs at the end of α-helix 3 [9]. View (B) illustrates the proximity of β-sheet 1 to the microtubule interaction surface of the head domain [10]. Eg5 structure was downloaded from NCBI-PubMed (www.pubmed.gov) and viewed using the UCSF Chimera program (www.cgl.ucsf.edu/chimera/). The structure spans aa 16-365, includes a Mg-ADP (red), and is missing several amino acids that were presumably disordered.

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