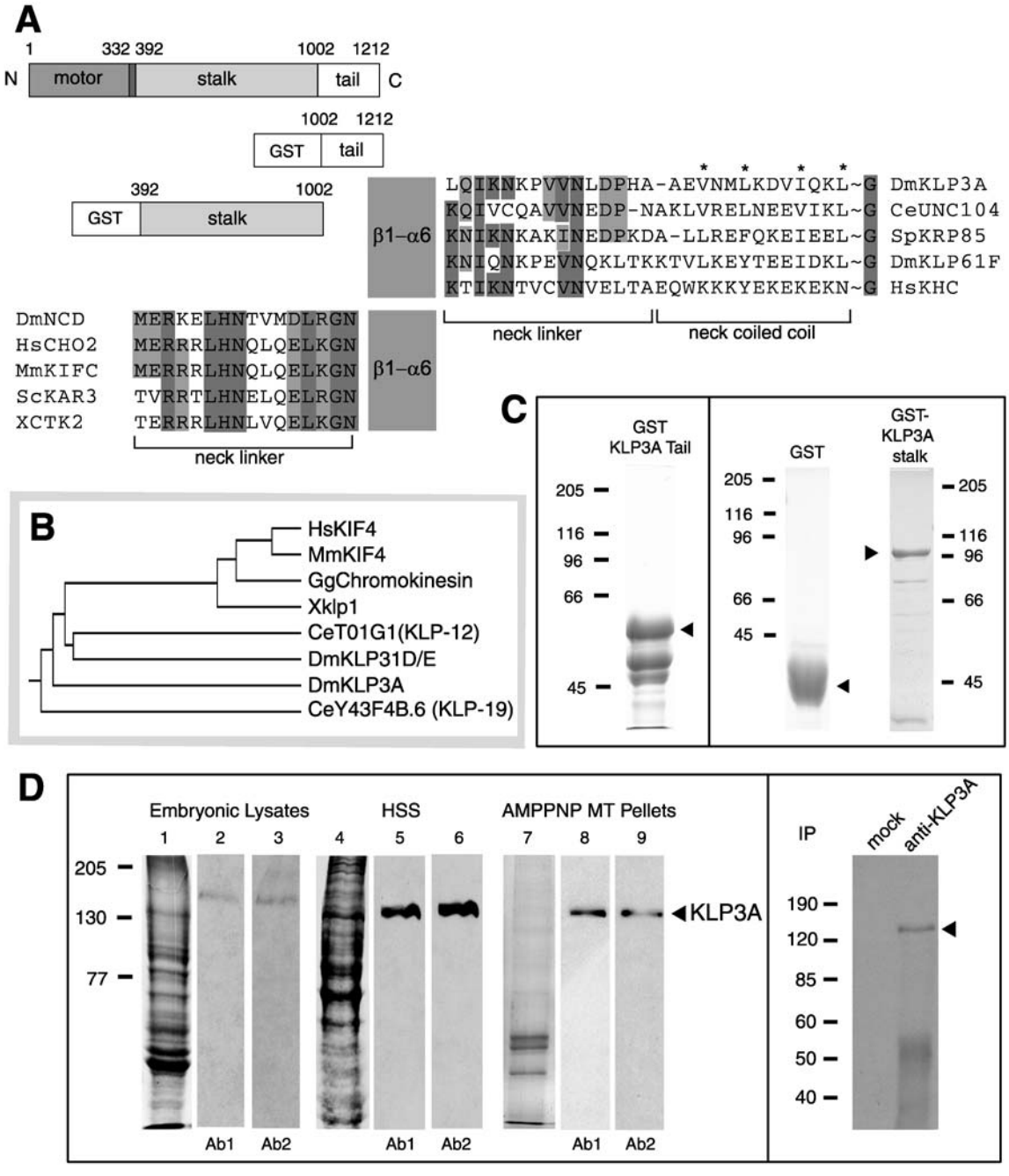
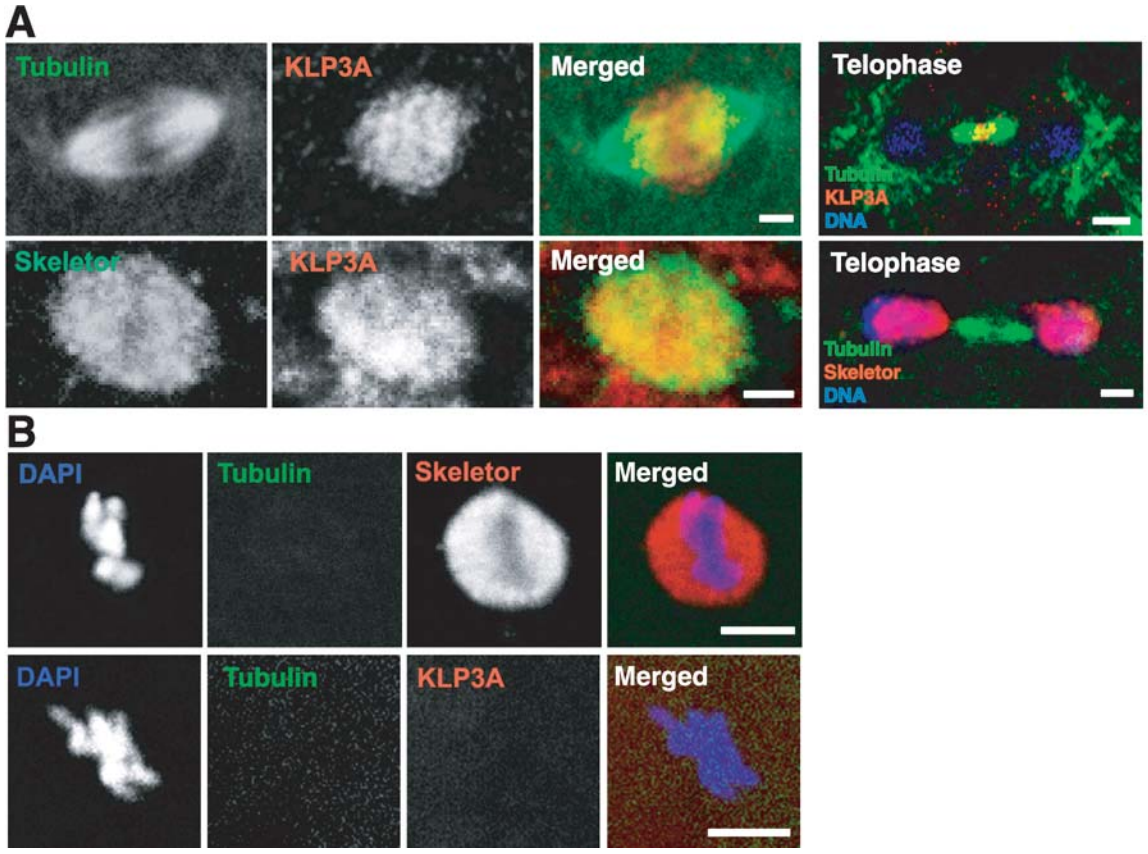
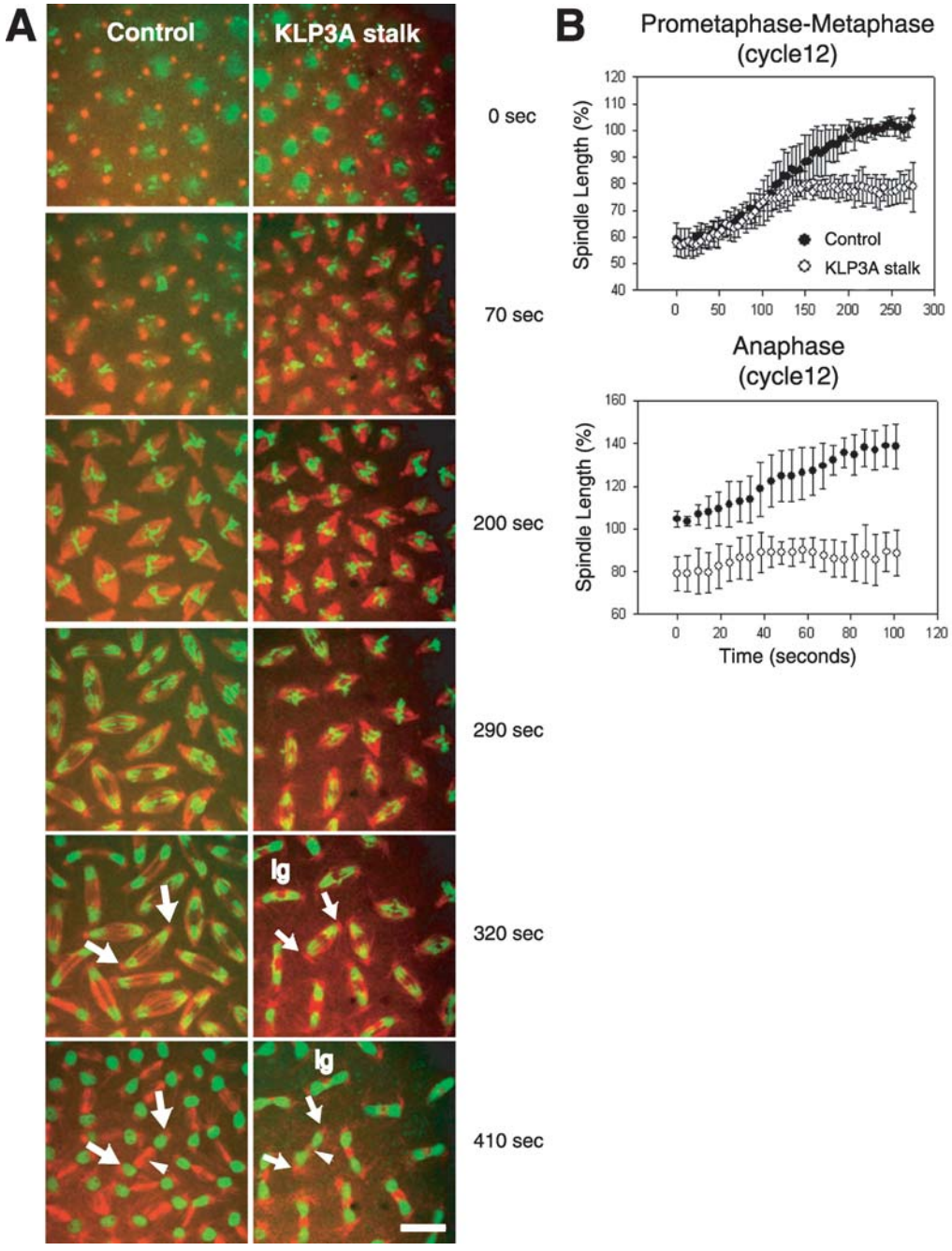

A
1
332 392
1002 1212
N
motor
stalk
tail
C
1002 1212
GST
tail
392
1002
GST
stalk
β1–α6
LQIKNKPVVNLDPHA-AEVNMLKDVIQKL~G DmKLP3A
KQIVCQAVVNEDP-NAKLVRELNEEVIKL~G CeUNC104
KNIKNKAKINEDPKDA-LLREFQKEIEEL~G SpKRP85
KNIQNKPEVNQKLTKKTVLKEYTEEIDKL~G DmKLP61F
KTIKNTVCVNVELTAEQWKKKYEKEKEKN~G HsKHC
neck linker
neck coiled coil
DmNCD MERKELHNTVMDLRGN
HsCHO2 MERRRLHNQLQELKGN
MmKIFC MERRRLHNQLQELKGN
ScKAR3 TVRRTLHNELQELRGN
XCTK2 TERRRLHNLVQELKGN
β1–α6
neck linker
B
HsKIF4
MmKIF4
GgChromokinesin
Xklp1
CeT01G1(KLP-12)
DmKLP31D/E
DmKLP3A
CeY43F4B.6 (KLP-19)
C
GST
KLP3A Tail
205
116
96
66
45
GST
GST-
KLP3A
stalk
D
Embryonic Lysates
HSS
AMPPNP MT Pellets
1 2 3 4 5 6 7 8 9
205
130
77
KLP3A
Ab1 Ab2 Ab1 Ab2 Ab1 Ab2
IP
mock
anti-KLP3A
190
120
85
60
50
40


A
Tubulin
KLP3A
Merged
Telophase
Tubulin
KLP3A
DNA
Skeletor
KLP3A
Merged
Telophase
Tubulin
Skeletor
DNA
B
DAPI
Tubulin
Skeletor
Merged
DAPI
Tubulin
KLP3A
Merged


A
Control
KLP3A stalk
0 sec
70 sec
200 sec
290 sec
320 sec
410 sec
lg
lg
B
Prometaphase-Metaphase
(cycle12)
Spindle Length (%)
Control
KLP3A stalk
Anaphase
(cycle12)
Spindle Length (%)
Time (seconds)

Supplementary Figure Legends

Figure S1. **Molecular Analysis and probes for KLP3A inhibition.** (A) Top, linear maps of KLP3A and the GST-tail and GST-KLP3A stalk fragments that were used as antigen and as a dominant negative construct, respectively. The numbers above the map correspond to the amino acids for each domain of the full-length KLP3A polypeptide. Bottom, comparison of conserved neck motifs in plus end directed KinN and minus end directed KinC motors, respectively (adapted from Vale and Fletterick, 1997). This alignment indicates that KLP3A and other KinN proteins share three characteristic neck motifs following the catalytic core, 1) the sequence K/RxIxNxxxV/IN, 2) several hydrophobic residues indicative of alpha helical coiled coil formation and 3) a glycine which may mark the beginning of a flexible hinge. In contrast, KinC motors have a conserved LxE/DLKGN preceding the catalytic core. (B) Phylogenetic tree showing that KLP3A is a member of the chromokinesin family. The tree was built from kinesin motor domain sequence alignment using Clustal method with PAM250 residue weight table (Megalign software). (C) Left panel, Coomassie-stained gel showing purified recombinant GST-KLP3A tail proteins that were used to raise rabbit polyclonal antisera against KLP3A. The fusion protein runs at ~50 kD and the lower molecular weight proteins are believed to be degradation products that are recognized by anti-GST antibody on the western blot (our unpublished results). Right panel, Coomassie-stained gel showing the purity of the ~100 kD GST-KLP3A stalk or ~27 kD GST (used as control) proteins. (D) Left panel, Immunoblots showing that both anti-KLP3A antibodies specifically recognize the KLP3A polypeptide (~140 kD). Lanes 1, 4, and 7 show total

Coomassie-stained proteins of 0-2 h *Drosophila* embryonic lysates (lane 1), high speed supernatants (HSS) (lane 4) and AMPPNP MT pellets (lane 7) separated by SDS-7.5% PAGE. Lanes 2, 3, 5, 6, 8, and 9 show immunoblots probed with the affinity purified anti-KLP3A tail antibodies from two rabbits. These blots were not loaded quantitatively, and therefore do not display the enrichment for KLP3A that occurs in the MT preparations. Right panel, Western blot showing that *Drosophila* embryonic KLP3A protein was immunoprecipitated by anti-KLP3A antibodies, as indicated by arrowhead (lower band, Ig heavy chain). Mock (protein A bead only) or anti-KLP3A immunoprecipitates were run on SDS-7.5% PAGE and probed with anti-KLP3A Abs.

Figure S2. **KLP3A displays MT-dependent association with embryonic spindles concentrating in an indistinct domain that partially overlaps with chromosomes and skeletor, prior to localizing in central spindles.** (A) High magnification images of metaphase embryonic spindles stained with anti-KLP3A and anti-tubulin or anti-skeletor (mAb1A1) antibodies. Colocalization of KLP3A and skeletor on the spindle is shown in yellow in metaphase spindles but not in telophase spindles with KLP3A in midbody and skeletor in nucleus. Bar, 2 μm. (B) 5mM colchicine-treated spindles triple labeled with DAPI (DNA), anti-α-tubulin and anti-KLP3A or anti-skeletor antibodies. MT depolymerization dissociates KLP3A but not skeletor from spindle remnants (some cytoplasmic KLP3A punctae remain after MT depolymerization, our unpublished results). Bar, 5 μm.

Figure S3. **Effects of microinjection of dominant negative GST-KLP3A stalk proteins on mitosis.** (A) Time-lapse confocal micrographs from embryos injected with GST (control) or GST-KLP3A stalk proteins, showing defects in the positioning of spindle poles and daughter nuclei following KLP3A inhibition. For KLP3A stalk injection, the images were taken from the region proximal to the injection site in the embryo (corresponding to region A, Fig. 2 C; see Online Supplemental Material, "Movie 7, KLP3A stalk injection"). Each panel indicates representative images from different time points corresponding to NEB, prometaphase, metaphase, anaphase A, anaphase B, and telophase with tubulin in red and chromosomes in green. Bar. 10 μm. (B) Plots showing spindle pole separation as a function of time for GST (control) and dominant negative KLP3A stalk- injected embryos.