Supplementary Materials

Abbreviations used: IPTG, Isopropyl β-D-1-thiogalactopyranoside; PMSF, Phenylmethylsulfonyl fluoride

Supplementary Methods

Plasmid Construction

The full-length Klp5 and Klp6 coding regions were generated by RT-PCR with *S. pombe* RNA using the following primers:

(Klp5 5' *Sap*I) : 5'-GGTGGTTGCTCTTCCACCATGTCAAGACAGTCGTCCATTACC-3', (Klp5 3' *Pst*I): 5'-GGTGGTCTGCAGTCATTAGTGATGATGATGGTGGTGGGTGGCTTTCTCTTCTTCGTTTTC-3',

(Klp6 5' *BamH*I): 5'- AGTACAGGATCCCCTTTCTATGG-3',

(Klp6 3' *Not*I): 5'- ATAAGTTTGCGGCCGCTCAAGCATTAGGAGTATTCTCAG-3'.

The resulting PCR-products were blunt-end cloned into the "pCR-Blunt II Topo"- vector (Invitrogen). The bacterial expression plasmid pKlp6MDN (aa1-440) containing the motor domain and neck (MDN) of this kinesin was obtained by PCR using the cloned full-length Klp cDNA in the pCR-Blunt II Topo vector as template. The 5'-primer (Klp6MDN5'-*Sac*I: 5'-GCATC*GAGCTC* ATG AAA GAA GGG TCT TCA ATT TCC-3') was complementary to the first 6 amino acids of the kinesin and introduced a *Sac*I restriction site. The 3'-primer

(Klp6MDN3'- *Not*I :5'-AAG GAA AAA AGCGGCCGC TTT TCG TAC CTC CTT ATT TAA AGC-3'), complementary to the DNA sequence corresponding to amino acid 433-440 of the kinesin,

was used to introduce a *Not*I restriction site. The resultant PCR product was cloned into the bacterial expression vector pET-21a(+) (Novagen) using *Sac*I & *Not*I restriction sites. This vector added an amino-terminal T7 epitope tag as well as a 6xHIS tag at the kinesin C-terminus to facilitate protein purification. The expressed plasmid produced a truncated Klp6 protein with a predicted molecular mass of 52,491 Da.

For expression of Klp6 full-length (FL) protein in insect cells, the Klp6 cDNA in vector Klp6 Blunt II Topo was subcloned by PCR into the baculovirus expression vector, pFastBac Dual (Invirtogen), using the following primers:
Klp6FL 5' SmaI: 5'-TCCCCCGGGGGAATGAAAGAAGGGTCTTC-3' and Primer
Klp6 3' NcoI: 5'-
CATGCCATGGCATGTTAGTGGTGATGGTGATGGTGAGCGGCAGCATTAGGAGTATTCTC
AGT-3'. The predicted molecular mass for the expressed Klp6FL protein was 88,831 Da.

For the expression of Klp5 full-length (FL) protein in insect cells, the Klp5 cDNA in vector Klp5 Blunt II Topo was subcloned by PCR into the baculovirus expression vector pVL1393-6xHis (Invitrogen) using the following primers:
KLP5FL-5': 5'-CGGGATCCATGTCAAGACAGTCGTCC-3'and
KLP5FL-3': 5'-ATAAGAATGCGGCCGCTCGGTGGCTTTCTCTTCTTCG-3'. The primers introduced a *BamH*I restriction site at the 5'-end of the sequence, and a *Not*I restriction site at 3'-end. The predicted molecular mass for the expressed Klp5FL protein was 100,505 Da.

Virus construction was performed by Abgent, Inc. (San Diego, CA). The production of high titer virus and subsequent infection of Sf9 cells was performed at the Tissue Culture Core Facility (University of Colorado Cancer Center, UCHSC at Fitzsimons, Aurora, Colorado). All PCR reactions for plasmid constructions were performed using a proofreading polymerase (*Pfu* polymerase, Invitrogen). The correct sequences of all constructs were verified by DNA sequencing.

Protein Expression and Purification

For the bacterial expression and purification of the truncated Klp6MDN protein, the *E. coli* strain Rosetta (DE3)pLysS (Novagen) was transformed with the plasmid pKlp6MDN (aa1-440). Cells were grown in LB media supplemented with 100 µg/ml carbenicillin and 80 µg/ml chloramphenicol at 37°C with shaking. Overnight cultures (1 liter) were diluted into 2 liters fresh media, incubated until the cultures reached an OD_{600} of ~1. Protein expression was induced by adding IPTG to a final concentration of 1mM. The cells were incubated at 18°C for 4h and collected by centrifugation at 4000 x g for 5min. Cells were washed once with Ni-NTA-lysis buffer (50 mM NaH_2P0_4 at pH 8.0, 500 mM NaCl, 10 mM imidazole,1 mM PMSF, 0.1% Tween, 0.1% Triton X-100). Cell pellets were frozen in liquid nitrogen and stored an -80°C. For cell lysis, cells were thawed in lysis buffer and briefly sonicated. After lysis, the whole extracts were clarified by centrifugation at 30,000 x g for 40 min at 4°C. The supernatant was incubated with Ni-NTA-Agarose (Qiagen) for 1.5h at 4°C with gentle rocking. The agarose/protein mixture was loaded into a chromatography column and washed with 1x column volume of lysis buffer, followed by 3x column volumes with wash-buffer 1 (50 mM NaH_2P0_4 at pH 8.0, 300 mM NaCl, 20 mM imidazole, 1 mM PMSF) and 2x column volumes with wash-buffer 2 (50 mM NaH_2P0_4 at pH 7.0, 150 mM NaCl, 40 mM imidazole, 1 mM PMSF). The protein was eluted with elution-buffer (50 mM NaH_2P0_4 at pH 7.0, 150 mM NaCl, 250 mM imidazole, 1 mM PMSF) by collecting 1 ml fractions. Fractions with the most protein were pooled and poured onto a desalting column (PD10 Bio-Rad) for buffer exchange (80mM PIPES at pH 6.9, 1mM $MgS0_4$, 1mM EGTA, 1mM DTT, 20mM KCl). Final protein samples were concentrated ~ 10 fold by centrifugation in Amicon centrifugal concentrators (Millipore), then frozen in liquid nitrogen and stored at -80°C.

For expression of full-length Klp5 and Klp6, Sf9s in suspension culture were infected with the above-described baculovirus expression vector and cultured at 27^{o}C in Grace's Insect Cell Culture Medium + 10% heat-inactivated fetal bovine serum + 0.1% Pluronic F68 + 25µg/ml

gentamicin. At the end of infection, the cells were harvested and washed once with PBS and then frozen at -80 °C. Pellets of frozen cells were thawed on ice, incubated with 25 ml lysis buffer (50 mM NaH_2P0_4 at pH 6.9, 300 mM NaCl, 10mM imidazole, 1% NP-40, and the protease inhibitor cocktail (PI cocktail): 10 μg/ml of each: aprotinin, leupeptin, soybean trypsin inhibitor, pepstatin; 0.25 mg/ml pefabloc SC, 80 μg/ml of both antipain-dihydrochloride and chymostatin plus 1 tablet from Roche (Cat No. 1836170) and 0.4% of a protease inhibitor cocktail from Sigma (P8849). Cells were disrupted with a dounce homogenizer, and the lysate clarified by centrifugation at 85,000 x g for 30min. The supernatant was incubated with Ni-NTA-Agarose (Qiagen) for 1.5h at 4°C with gentle rocking. The agarose/protein mixture was poured into a chromatography column and washed with 1x column volume of lysis buffer, followed by 10 column volumes of wash-buffer A (50mM NaH_2P0_4 at pH 6.9, 500mM NaCl, 10mM imidazole plus the PI cocktail) and then 4x column volumes with wash-buffer B (50mM NaH_2P0_4 at pH 6.9, 150mM NaCl, 75mM imidazole plus the PI cocktail). Protein was eluted from the column with buffer E (50mM NaH_2P0_4 at pH 6.8, 150mM NaCl, 250mM imidazole plus the PI cocktail). The peak fraction was then loaded onto a 5-20% sucrose gradient in NaCl buffer (25 mM NaH_2P0_4 at pH 6.8, 0.5 M NaCl, 1 mM EDTA plus the PI cocktail) and centrifuged at 36,000rpm (Beckman SW 41 rotor) for 20h at 4°C. Sucrose gradient fractions were collected, then frozen in liquid nitrogen and stored at -80°C. Full-length Klp6 was prepared as described for co-expressed Klp5FL/Klp6FL above with the following buffer modifications: lysis buffer (50 mM NaH_2P0_4 at pH 8.0, 300 mM NaCl, 10mM imidazole, 1% NP-40, 10% glycerol, 1 mM B-mercaptoethanol (BME), 0.5 mM MgATP and the PI cocktail); wash-buffer A (50mM NaH_2P0_4 at pH 8, 500mM NaCl, 10mM imidazole, 0.1% NP-40, 1 mM BME, 10 μM MgATP plus the PI cocktail described above); wash-buffer B (as described above plus 0.1% NP-40, 1 mM BME and 10 μM MgATP); elution buffer (as described above plus 0.1% NP-40, 1 mM BME and 10 μM MgATP).

Hydrodynamic Characterization

The full-length Klp5/6 expressed in insect cells and bacterially-expressed Klp6MDN were characterized by sucrose gradient centrifugation and size-exclusion chromatography to measure their molecular masses in solution. Sedimentation was performed at 4°C on a 5-20% sucrose gradient at 36,000 rpm (Beckman SW 41 rotor) for 20h. Full-length Klp5/6 was spun in NaCl buffer (25 mM Sodium Phosphate at pH 6.8, 0.5 M NaCl, 1 mM EDTA plus the complete protease inhibitor cocktail); Klp6MDN was characterized in BRB80 Buffer (80mM PIPES, 1mM $MgCl_2$, 1mM EGTA supplemented with 25mM KCl and 1mM MgATP). Protein standards with known $S_{20,w}$ values were used to calibrate the gradient. Gel filtration of full-length Klp5 and Klp6 was performed on a Superdex 200™ 10/300 GL column using an ÄKTA FPLC system (GE); Klp6MDN was characterized on a Sephacryl S-300 column (Pharmacia). Gel Filtration molecular weight makers (Bio-Rad) were run on the same columns as calibration standards. Ion-exchange chromatography was performed on full-length Klp5/6 in 50 mM $NaHPO_4$ at pH 6.9 using a SP Sepharose XL cation exchange column, eluted with a linear gradient of 0 to 1 M NaCl in column buffer. Protein was detected and characterized by SDS gel electrophoresis and Coomassie staining or by Western blotting using anti-His antibody (Penta His-Ab, Qiagen).

Steady State ATPase Assays

To determine ATPase activities we used a coupled enzyme ATPase assay described on the Kinesin homepage: http://www.proweb.org/kinesin/Methods/ATPase_assay.html, as modified from Huang and Hackney JBC 269, 16493-501, 1994. Purified Klp protein was mixed with taxol-stabilized microtubules and assay buffer (final concentrations of buffer: 50mM Tris-acetate at pH 7.5, 1 mM $MgCl_2$, 1mM DTT supplemented with 1mM MgATP, 3mM phosphoenolpyruvate, 0.2mM NADH and Rabbit muscle PK/LDH (Sigma) in 50% glycerol). The sample was mixed and transferred to a plastic cuvette, and the change in absorbance was measured in a spectrophotometer at 6 sec intervals for 5-10 min. The change in OD_{340}

per minute was used to determine the $k_{cat}(s^{-1})$. The motor protein without microtubules and microtubules without motor protein were used as baseline controls. These values were subtracted from the corresponding values of motor protein and the same concentrations of microtubules. The measured rates (S^{-1}) were plotted against microtubule or MgATP concentrations and the data points were fit with the Michaelis-Menton equation using GraphPad Prism software to determine V_{max} and K_m values.

Supplemental Figures

Figure S1. Preparation of Klp6FL.

A*) Coomassie-stained SDS gel (A) and western-blot with penta-His antibodies (B) of the Klp6FL purification.* S: whole protein extract, FI: Flow-through, wash A and wash B fractions as described in supplemental methods. Eluted 2-4: Ni-NTA elution fractions. Sucrose Gradient fractions: 13-15. K5/K6: purified Klp5FL/Klp6FL as a loading control. M: molecular weight standards (values given in kDa).

Figure S2. Hydrodynamic characterization of Klp6MDN.

A) *Sucrose gradient fractionation of Klp6 MDN and protein standards*. Upper panel: SDS-gel of fractions from the sedimentation of Klp6MDN on a sucrose gradient. Bottom panel: Corresponding SDS-gel of the protein standard fractions with known $S_{20,w}$ values. M: molecular weight standards (values given in kDa). The $S_{20,w}$ value for Klp6MDN = 5.6 S under the described conditions (see Materials & Methods).

B) *Determination of the diffusion constant for Klp6MDN*. The diffusion constant, $D_{20,w}$, of Klp6MDN was obtained by gel filtration chromatography on a Sephacryl S-300 column. The inset shows the SDS-gel of the column elution profile fractions 41-53. The $D_{20,w}$ value for

Klp6MDN was 4 x10^{-7} cm^2/s at the described conditions, using the least-squares fit of K_{av} values for the standard proteins plotted versus their $1/D_{20, w}$ values. The native molecular weight of Klp6MDN was 118 kDa, as calculated from the Svedberg equation.

Figure S3. ATPase activity of Klp6MDN.

Enzyme activity as a function of MT concentration. Assay conditions: 100 nM Klp6MDN, 1 mM MgATP and taxol-stabilized MTs (0-1.2 μM tubulin dimmers). K_{cat} with saturating concentrations of MTs was 0.93 ± 0.01 s^{-1} and $K_{M, MT}$ 0.06 ± 0.004 μM.

Figure S4. Sedimentation analysis of MT depolymerization with Klp5/6FL and MCAK.

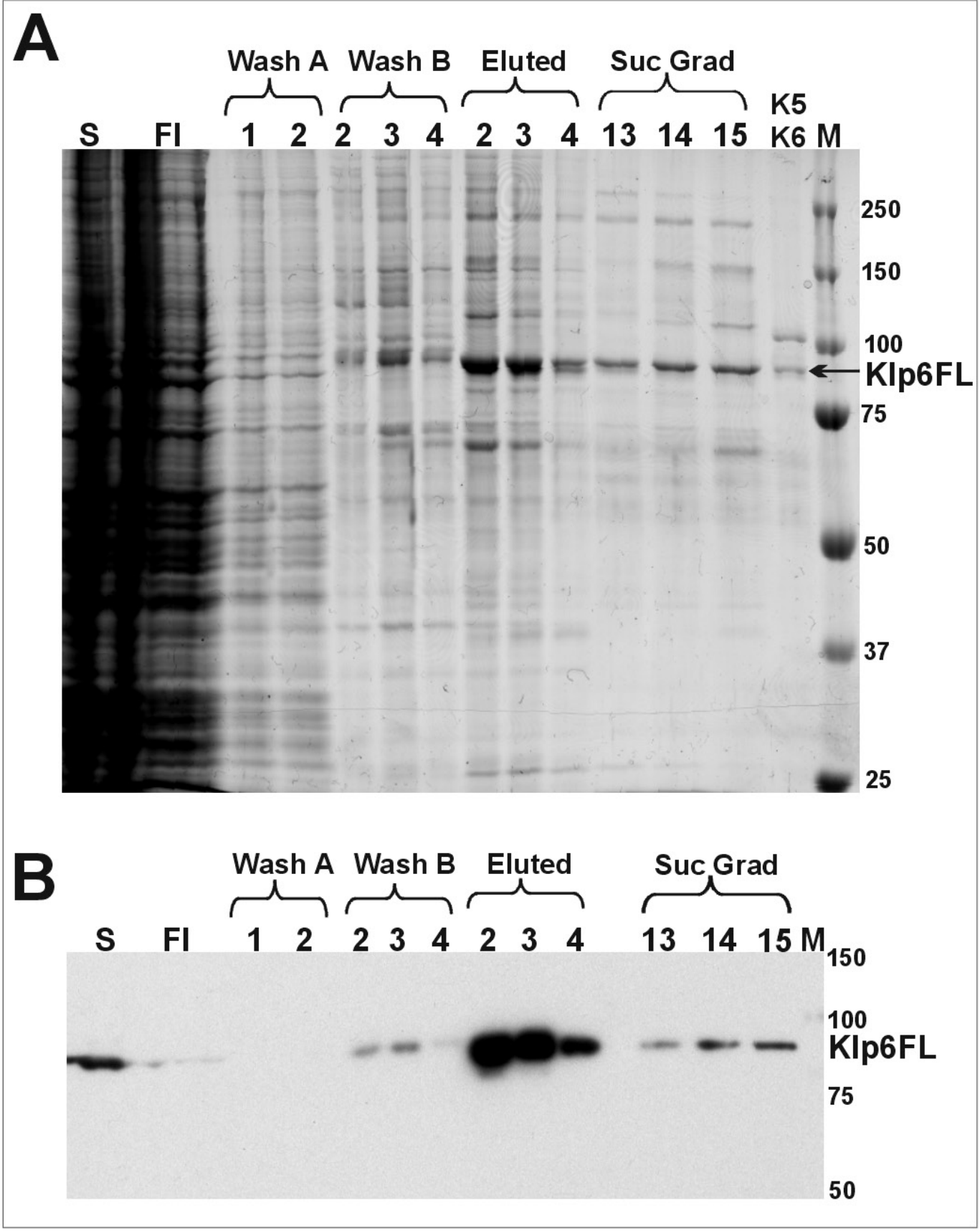
Taxol-stabilized MTs (2.5 μM tubulin) were mixed with: 1) 50 nM Klp5/6FL in buffer (BRB80 plus 70mM KCl, 2 mM MgATP and 3 μm Taxol), 2) 200 nM MCAK (a kind gift from Claire Walczak) in the same buffer, or 3) buffer only. All samples were incubated for 30 min at room temp and then centrifuged in a Beckman Airfuge at 20 psi for 10 min. The corresponding supernatants (S) and pellets (P) were analyzed by SDS-page. M: molecular weight standards (values given in kDa).

Movie S1: Klp5/6FL is a plus-end directed microtubule motor.

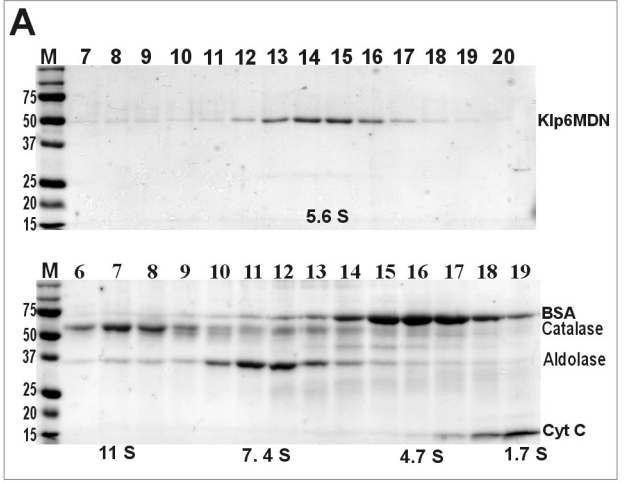
A movie of polarity marked Fl/Rh/MTs (fluorescent seed and rhodamine ends) in the presence of Klp5/6FL moving with their minus ends (shorter Rh segments) leading. The first frame shows a superposition of images taken with the FITC and Texas Red filter cubes; subsequent images show the Texas Red images only. The movie is played 240x faster than recorded. Scale bar: 4 μm.

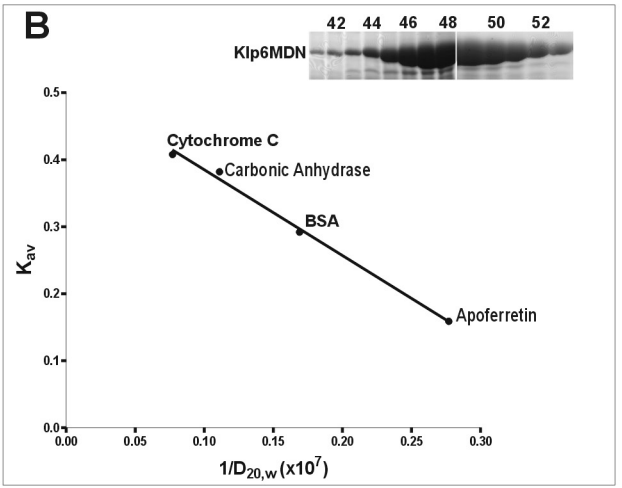
Movie S2: Klp5/6FL coated beads move with the disassembling MT.

MTs were grown by nucleating the growth of purified bovine tubulin with *Chlamydomonas axonemes* using standard assembly conditions. They were then capped with Rh-GMPCPP tubulin and incubated with 0.5 μm polystyrene beads coated with Klp5/6FL. Following the photo-induced depolymerization of the MT cap, the two attached beads moved along the depolymerizing MT for 17 μm at a speed of 13 μm /min. Note the absence of rotational motion as the beads move. Images were taken using DIC optics. The movie is played 4.5x faster than recorded.



Supplemental Figure 1: Grissom et al.





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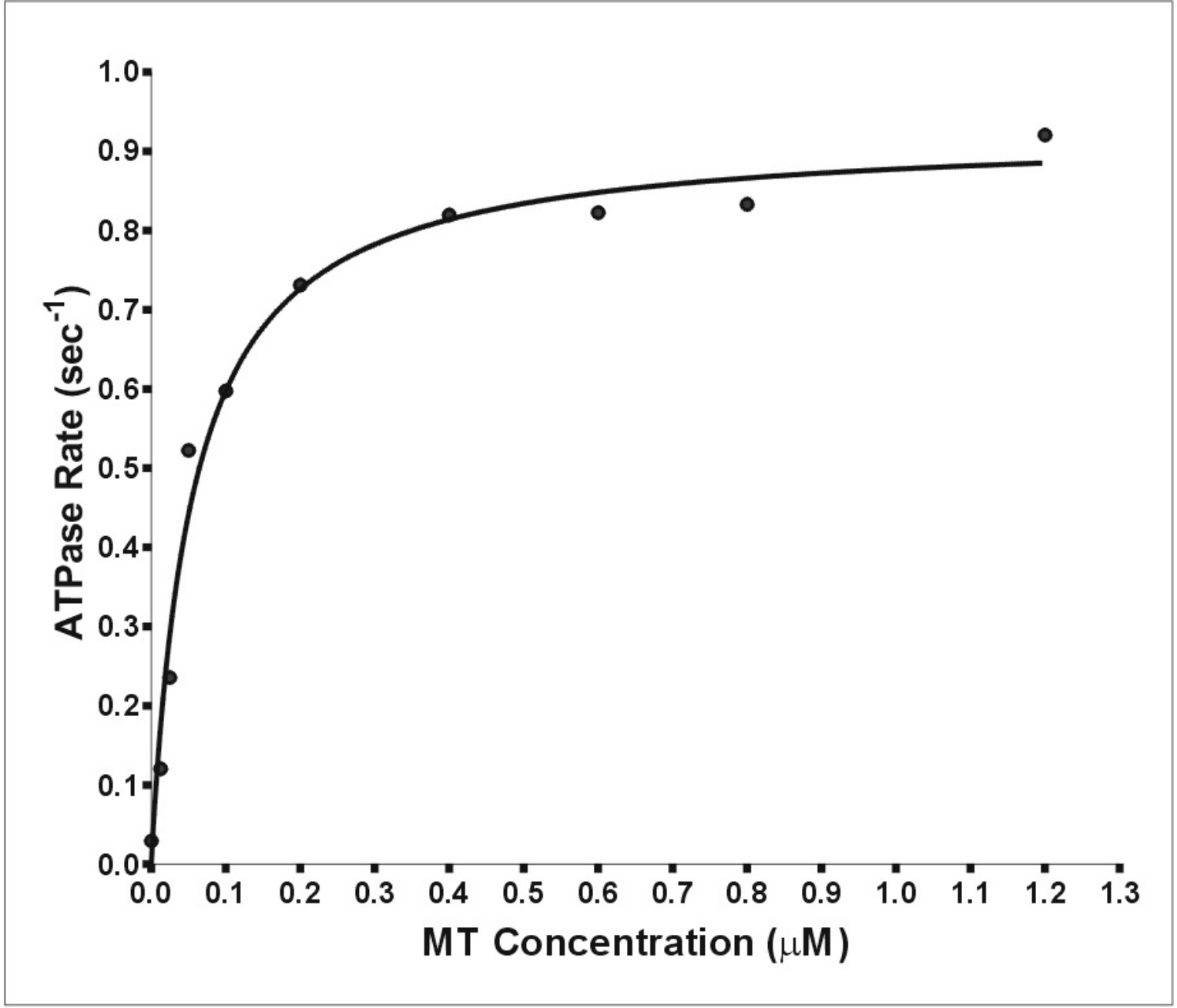
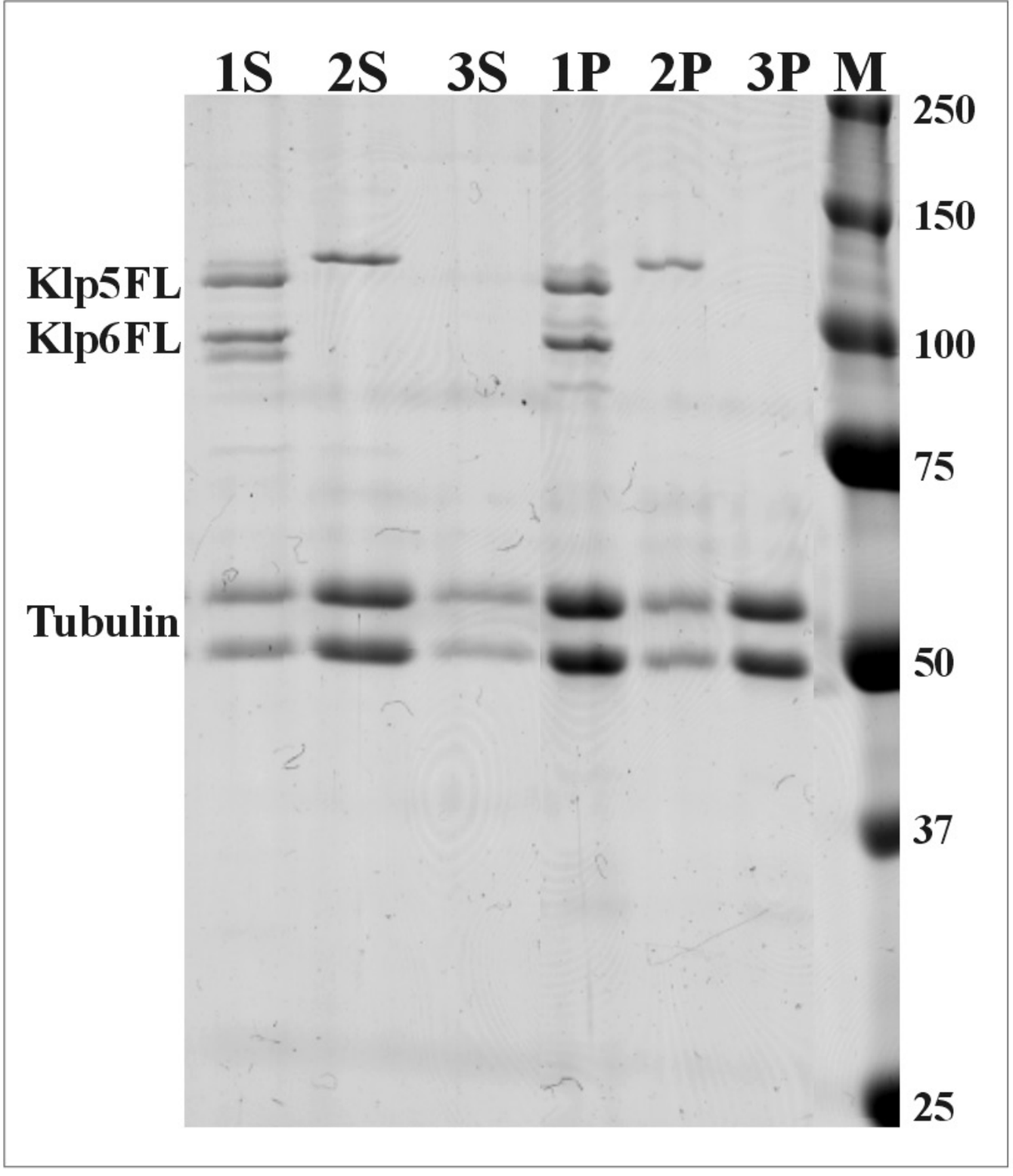


Supplemental Figure 3: Grissom et al.



Supplemental Figure 4: Grissom et al.