Kinetic and mechanistic basis of the non-processive Kinesin-3 motor NcKin3

Sarah Adio¹, Marieke Bloemink², Michaela Hartel¹, Sven Leier¹, Michael A. Geeves², Günther Woehlke¹,*

¹ Institute for Cell Biology, Ludwig-Maximilians-University Munich, Schillerstr. 42, D-80336 Munich, Germany, ² Department of Biosciences, University of Kent, Canterbury, Kent CT2 7NJ, UK

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*Corresponding author: Günther Woehlke, phone: +49 (89) 2180 75889, fax: +49 (89) 2180 75882, email: guenther.woehlke@lrz.uni-muenchen.de
Kinesin-3 motors have been shown to transport cellular cargo along microtubules and to function according to mechanisms that differ from the conventional hand-over-hand mechanism. To find out whether the mechanisms described for Kif1A and CeUnc104 cover the full spectrum of Kinesin-3 motors, we characterize here NcKin3, a novel member of the Kinesin-3 family that localizes to mitochondria of ascomycetes. We show that NcKin3 does not move in a K-loop-dependent way as Kif1A, or in a cluster-dependent way as CeUnc104. Its in vitro gliding velocity ranges between 0.30 and 0.64 μm/s and correlates positively with motor density. The processivity index (k_{bi,ratio}) of ~3 reveals that not more than 3 ATP molecules are hydrolysed per productive microtubule encounter. The NcKin3 duty ratio of 0.03 indicates that the motor spends only a minute fraction of the ATPase cycle attached to the filament. Unlike other Kinesin-3 family members, NcKin3 forms stable dimers but only one subunit releases ADP in a microtubule-dependent fashion. Together, these data exclude a processive hand-over-hand mechanism of movement, and suggest a power-stroke mechanism where nucleotide-dependent structural changes in a single motor domain lead to displacement of the motor along the filament. Thus, NcKin3 is the first plus-end directed kinesin motor that is dimeric but moves in a non-processive fashion to its destination.

The kinesin superfamily of proteins contains molecular motors that move along microtubules. Members of the founding class, Kinesin-1 (conventional), move according to the so-called hand-over-hand mechanism that leads to processive motility (1). This mechanism involves two motor heads that alternately bind to the microtubule and produce a stepwise progression of the motor. It implies that the catalytic cycles of the motor heads alternate because the nucleotide state of the catalytic domain determines the microtubule affinity (2). This mechanism is referred to as alternating site catalysis, and has been proven by measuring the consecutive release of ADP from head 1 and 2 of the kinesin dimer (3-5). ADP release experiments show that half of the enzyme-bound ADP is liberated upon interaction with the microtubule, and the other half upon addition of ATP. Afterwards, the motor continues to perform processive catalytic cycles due to the interlaced interaction of both motor heads. In agreement, the rate of the second ADP release is at least as fast as the stepping rate. The kinetic model of alternating site catalysis is consistent with the hand-over-hand mechanism that predicts shifted phases of the kinetic cycles of both motor heads. All Kinesin-1 motors investigated so far conform to this model.

Vice versa, the non-processive Kinesin-14 motor Ncd does not show the ADP release pattern typical for alternating site catalysis. Ncd is a homodimeric kinesin from Drosophila melanogaster, and involved in mitosis and meiosis (6-8). It moves to the microtubule minus-end, and most likely generates motility due to a molecular power-stroke (9-15). ADP release also occurs in two steps, one that is triggered by microtubule binding, and one that is induced by nucleotide binding. By contrast to conventional kinesin, however, Ncd dissociates from the microtubule at a rate in the range of k_{cat}, implying that the motor dissociates after one kinetic cycle. The ADP release behavior of Kinesin-1 and Ncd therefore reveal two opposing motility mechanisms, namely processive hand-over-hand motility and non-processive powerstroke motility. These types of motility, however, do not cover the entire spectrum of kinesin mechanisms. The Kinesin-3 type motor KIF1A has been reported to move continuously along microtubules in a quasi-processive manner, although the constructs used were monomeric (16-20). These truncated motor constructs, too, change their microtubule affinity in a nucleotide-dependent fashion, but do not dissociate from the filament in their weak microtubule-binding state. They do not avoid dissociation by alternating microtubule interactions but by the action of a positively charged, lysine-rich region (K-loop). This loop binds to the negatively charged, glutamate-rich C-terminus of tubulin, the E-hook. This interaction allows lateral sliding of Kinesin-3 proteins to the next binding site, and prevents detachment from the microtubule.

It is controversial whether all Kinesin-3 motors work as described for KIF1A. Dictyostelium discoideum DdUnc104, for example, is constitutively dimeric, and thus could move in
principle by a hand-over-hand mechanism (21). On the other hand, *Caenorhabditis elegans* CeUnc104 has been reported to move non-processively (22,23). Interestingly, CeUnc104 becomes a processive motor if dimerization is enforced either by high local motor concentrations, or by insertion of an artificial dimerization domain (23). In vivo the switch between the monomeric and the dimeric state might regulate cargo transport (24). Remarkably, *C. elegans* or *D. discoideum* Unc104 have not been investigated in stoichiometric or kinetic ADP release assays that might support or disprove half-site catalysis. All KIF1A and KIF1C constructs that have been characterized previously were monomeric (25). Therefore, half-site catalysis cannot occur.

Given the fundamental functional differences among different Kinesin-3 motors, it is interesting to know whether this class of kinesins is based on a common underlying mechanism. If not, it is interesting why phylogenetically related proteins developed divergent mechanisms. To address these questions, we cloned a novel Kinesin-3 member, NcKin3 from *Neurospora crassa*, which is involved in transporting and shaping mitochondria (26). We show that NcKin3 is a dimeric, plus-end directed microtubule motor whose gliding velocity correlates positively with the motor density on the coverslip. Comparison of *K*<sub>M,ATP</sub> values in gliding and ATPase assays indicates a low duty ratio. This, together with a low processivity index (*k*<sub>bi,ratio</sub>), suggests that NcKin3 is not processive. In agreement, the motor detaches at a rate comparable to the steady state ATP turnover rate from the filament. Remarkably, only one of the motor heads releases ADP in a microtubule-dependent fashion. The second head loses ADP at a constant, microtubule-independent rate. Together, our study presents the first description of a non-processive, plus-end directed kinesin vesicle motor.

**Experimental Procedures**

*Cloning, Protein expression and purification*—The DNA encoding NcKin3 was isolated from a mycelial cDNA library of *N. crassa* (27) (NCU03715.2 in *N. crassa* genome release 7, http://www.broad.mit.edu/annotation/fungi/neurospora_crasa_7/). For recombinant expression NcKin3 was cloned into a pT7.7 vector (28). A C-terminal truncated versions of NcKin3, consisting only of 558 amino acids (NcKin3-558) was generated from pT7.7-NcKin3 by PCR. A short peptide sequence was added to NcKin3-558 that adds a reactive cysteine to the protein. This allows labeling with maleimide compounds (PSIVHRKCF, (29)). At the C-terminus of NcKin3-558 an NgoMIV restriction site was inserted for the addition of amino acids 432 to 546 of human kinesin tail (hTail). The hTail DNA was transferred from a NcKin3hTail pT7-based plasmid (30) and inserted into pT7.7-NcKin3-558 via the NgoMIV and HindIII restriction sites.

For protein expression plasmids were transformed into BL21 Codon Plus (DE3)-RIL (Stratagene Inc., San Diego, USA). Cells were grown in ampicillin and chloramphenicol containing TPM medium (20g/l tryptone, 15g/l yeast extract, 2.5g/l Na<sub>2</sub>HPO<sub>4</sub>, 1g/l NaH<sub>2</sub>PO<sub>4</sub>, 10 mM glucose). Expression was induced at an OD<sub>580</sub> of 0.4 to 0.6 with 1 mM isopropyl-β-D-thiogalactopyranoside and incubated over night at 22°C. Cells were harvested and stored at −70°C.

Proteins were purified by ion exchange chromatography on a High Trap SP Sepharose column as described (Fig. 1B) (31). Peak fractions were supplied with 10% glycerol, frozen in liquid nitrogen and stored at −70°C. Protein concentration was estimated by a Bradford assay (Bio-Rad Laboratories, Hercules, CA, USA) and via the absorption at a wavelength of 280nm.

**Determination of the oligomerization state**—To characterize the oligomerization state of NcKin3 the Svedberg coefficient and the Stokes’ radius were determined by density gradient centrifugation and gelfiltration (32). The molecular weight was calculated according to (33):  

\[ M_r = \frac{s_{20,w} n_A^* 6 \pi \eta \rho_{stokes}}{(1 - \nu^* \rho)} \]

where *n*<sub>A</sub> is Avogadro’s number, *η* viscosity (10<sup>3</sup> Nms<sup>−2</sup>), *ν* specific volume of the sample (0.725 cm<sup>3</sup>/g) and *ρ* is the density of the medium (1.0 g/cm<sup>3</sup>).
The reaction was stopped by addition of 10 mM DTT. Active motors were selected in a microtubule binding and release step (34).

A flow cell was incubated with 1mg/ml streptavidin (Sigma-Aldrich Co., St. Louis, MO, USA) in BRB80+ (80 mM PIPES-KOH pH 6.8, 5 mM MgCl₂, 1 mM EGTA), which was filled with biotin-labeled NcKin3 in motility buffer (10 mM MgCl₂, 10 mM ATP, 100 mM KCl, 20 µM paclitaxel, 1 mg/ml BSA, 0.8 mg/ml casein in BRB80+) after washing with blocking buffer (1 mg/ml BSA, 0.8 mg/ml casein in BRB80+), incubated for additional 5 min and filled with microtubules in motility buffer. Gliding of the microtubules was observed in a Zeiss Axiophot using video enhanced phase-contrast microscopy at 22°C, and analyzed manually (32).

To measure the concentration dependence of the velocity, the hTail-tagged version of NcKin3-558 was used. Fluorescently labeled microtubules were observed in a total internal reflection microscope and their velocity was measured using manufacturer software (Olympus Biosystems, Planegg, Germany). For statistical analysis Kaleidagraph 3.6 (Synergy Software, Reading, PA, USA) and Prism 4.0 (GraphPad Software Inc., San Diego, USA) were used.

**Basal ATPase activity**-Slow steady-state ATPase rates in the absence of polymerized tubulin were measured using [γ-32P]ATP (35). NcKin3 (5 µM) was incubated in 12A25+buffer (12.5 mM Aces-KOH, 25 mM Potassium acetate, 5 mM MgCl₂, 0.5 M EGTA, pH 6.8) with 1 mM [γ-32P]ATP at 22°C. After various time points the reaction was quenched in 0.3 M perchloric acid. A suspension of charcoal in 1 mM NaH₂PO₄ was added, mixed and centrifuged in order to separate the charcoal. After determination of the specific activity of the [γ-32P]ATP solution, [γ-32P]-labeled inorganic phosphate in the supernatant was quantified by scintillation counting. The ATPase rates were calculated from the hyperbolic fits of the time traces.

**Steady-state ATPase**-Microtubule activated steady-state ATPase rates were determined in a coupled enzymatic assay (36). The assay was performed in 12A25+buffer at 22°C. K₀.₅₅₅₅ was determined at an ATP concentration of 1 mM. The microtubule concentration was maintained at approximately 10 µM for titrations with ATP. NcKin3 concentrations were typically 1 µM. Tubulin was purified from pig brain as described (37). Microtubules were obtained by spinning a freshly thawed tubulin aliquot at 120,000 g and 4°C, supplementing the supernatant with 1 mM GTP and 20 µM paclitaxel, and removing excess nucleotides by centrifugation through a sucrose cushion. Finally, the microtubule pellet was suspended in 12A25+, 20 µM paclitaxel, and the protein concentration determined at 280 nm (3).

**Stochiometry of mant-ADP release**-NcKin3 was incubated with equimolar amounts of mant-ATP for 30 min on ice. The final kinesin concentration in the assay was 0.9 µM. The fluorescence decrease caused by mant-ADP release was observed in an Aminco Bowman Spectrofluorimeter (32). The fluorescence was excited at 356 nm, the emission detected at 445 nm. All experiments were performed at 22°C in 12A25+ buffer. Microtubules were treated with apyrase prior to the centrifugation through a sucrose cushion. Microtubule concentrations ranged between 1 and 11 µM. To release mant-ADP from the second head an excess of unlabeled ATP (1 mM) was added subsequently.

**Pre-steady-state mant-ADP release**-NcKin3 was incubated with a four-fold excess of mant-ATP for 30 min on ice. Excess nucleotide was removed by gel filtration in Sephadex G-25 spin columns. The labeling ratio was calculated from the extinctions at 280 nm (ε = 38,000 M⁻¹cm⁻¹) and 356 nm (ε = 5,800 M⁻¹cm⁻¹). Pre-steady state mant-ADP release was measured in a BioLogic stopped-flow apparatus (SFM-3; BioLogic Inc., Grenoble, France) at 22°C in 12A25+ buffer. Fluorescence was excited at 365 nm, emission was detected at 442 nm using a band pass interference filter of ± 10 nm width (Chroma Technology Corp., Rockingham, VT, USA). A final concentration of 400 nM of mant-ADP-labeled NcKin3 was rapidly mixed with 0 to 12 µM microtubules in the presence of 1 mM ATP (final concentrations). The traces shown are averages of at least five stopped-flow traces. All traces obeyed single exponential behavior and were fitted using either the software BioKine (BioLogic Inc., Grenoble, France) or TableCurve2D (Systat Software Inc., Point Richmond, CA, USA). The rates showed a hyperbolic dependence on the microtubule
concentration and were fitted according to the equation:

\[ y = B + \frac{k_{\text{max}}[M]}{[M]+K_{0.5,M}} \]

The constants presented in this work are averages from three independent experiments.

**Cosedimentation Assay** Increasing amounts of NcKin3-558 (1 µM to 25 µM) in BRB80+ buffer were mixed with 2 µM microtubules in the presence of 1 mM ADP or 1 mM AMP-PNP. The samples were mixed and then centrifuged in a Beckmann Optima 100 ultracentrifuge (100,000 rpm, 10 min, 4°C). The supernatants were carefully removed and mixed with SDS sample buffer. The pellets were resuspended in BRB80+ and stopped in PAGE sample buffer. Pellet and supernatant samples were separated on 12%-SDS-PAGEs and stained with Colloidal Blue Staining (Invitrogen GmbH, Karlsruhe, Germany). Gel photos were taken with the EagleEye CCD camera system (Stratagene Inc., La Jolla, CA, USA) and quantified using the ImageJ software (http://rsb.info.nih.gov/nih-image/). The concentrations of motors in pellet and supernatant were analyzed with SigmaPlot Software (Systat Software, Inc., Point Richmond, CA, USA). Binding parameters were calculated from a hyperbolic curve fit.

**Detachment Rates** The microtubule detachment kinetics of NcKin3 was determined by the change of light scattering in two assays. (i) For stopped-flow assays, the NcKin3 microtubule complex was formed by incubating NcKin3-558 with a 1.5-fold excess of microtubules in the presence of 0.01 U/ml apyrase (Sigma-Aldrich Co., St. Louis, MO, USA). Subsequently, 0.4 µM of the complex (final concentration) was mixed with ATP (0 to 64 µM) in a stopped-flow apparatus (BioLogic Inc., Grenoble, France). The sample was illuminated at a wavelength of 436 nm and the light scattering signal observed through a 440 ± 10 nm band-pass filter. At least five traces of each ATP concentration were used for averaging. Averaged traces were analyzed by single exponential curve fitting using the TableCurve2D software (Systat Software Inc., Point Richmond, CA, USA). The observed rates were plotted against the ATP concentration and fitted according to the following equation:

\[ y = k_{\text{max, binding}} + \frac{k_{\text{max, detachment}}[\text{ATP}]}{([\text{ATP}]+K_{0.5,\text{ATP}})} \]

Detachment of NcKin3 from the microtubule was also (ii) measured via the light scattering signal in a flash-photolysis apparatus, described in detail in the supplement.

**Results**

**Unusual structural features of NcKin3** NcKin3 was found at mitochondria of the ascomycete N. crassa, suggesting that it is an organelle motor, and responsible for transport and shaping of mitochondria (26). To study the mechanistic basis of this motor, we isolated the DNA from a mycelial cDNA library and cloned it into a bacterial expression vector (27,38). The coding sequence comprises 1947 bp that code for a protein of 647 amino acids and a predicted molecular weight of 72 kDa. Noteworthy, the cDNA sequence comprises a 5’ extension that codes for 37 N-terminal amino acids that are not part of the conserved motor domain (Fig. 1). The NcKin3 motor domain sequence reveals a clear membership in the Kinesin-3 subfamily (39). However, the molecule shows several unusual features. For example, BLAST searches with the N-terminal extension in the Gen/EMBL database fail to detect any similarities outside fungal Kinesin-3s. The functional role is unclear because a truncated mutant (amino-acids 38-558) of NcKin3 lacking the extension shows the same microtubule-activated steady-state ATPase rate and half maximal activation constant for microtubules as the construct with the extension (amino acid 1-558, data not shown). As another distinctive feature, NcKin3 and other short fungal Kinesin-3 motors possess a much shorter and less charged K-loop region (typically 1 Arg and 1 Lys residue, separated by 2-3 residues; Fig. 1A). Finally, the C-terminal non-motor part is unusually short (about 200 amino acids), does not contain FHA or PH domains, and does not show homology to other Kinesin-3 clades. Curiously, the last 90 residues form a cluster that contains 21 Asp and 7 Glu residues that result in a highly negative net charge of the tail.
The recombinant full-length protein was active in ATPase and gliding assays but poorly expressed. Also, the preparation contained significant degradation products. Therefore, we cloned truncated versions lacking parts of the C-terminus (Fig. 1B). The longest truncation, NcKin3-558 was used in most experiments described here, and a reactive cysteine was added at the C-terminus, allowing modifications with maleimide labels (29,30). The truncated, biotin-tagged NcKin3-558 kinesin was equally fast in gliding assays as the recombinant full-length NcKin3 protein.

NcKin3 is a dimer-Automatic and manual analysis of the NcKin3 stalk sequence predicts the formation of a two-stranded coiled coil between residues 437 and 513, although some heptads deviate significantly from the ideal repeat pattern, and the start of the helix is uncertain (Fig. 1A). Coiled coils contain characteristic periodic repeats of seven amino acid residues numbered a-g, where positions a and d are mostly hydrophobic, and e and g are often charged or hydrophilic (40-42). In the case of NcKin3 the start of the coiled coil can be predicted as early as at residue 427 but then a change of the coil phase would occur after residue 441 (shift from a to f position).

In agreement with the prediction, gel filtration and sucrose density gradient centrifugation assays revealed that NcKin3 is dimeric (Fig. 2) (43). The two longest constructs, NcKin3-558 and -513 are stable dimers, suggesting that the full-length protein is also dimeric. Shorter constructs (NcKin3-415, -434, -457, -470 and -488) are monomeric, implying that the last 3 predicted coil heptads are crucial for dimer stability (Fig. 1). Vice versa, the first seven predicted heptads are not sufficient for dimerization in truncated constructs but, as shown below, are still functionally important. With the exception of D. discoideum Unc104, all other Kinesin-3 motors have been reported to be primarily monomeric (21-23,44,45). As NcKin3 does not show similarity to DdUnc104 outside the motor domain, it contains a so far unknown dimerization domain.

Features of NcKin3 motility-To find out whether NcKin3 is a microtubule motor we performed multiple motor gliding assays. At saturating motor densities on the surface of the flow chamber microtubules were transported with a maximum velocity of 0.59 ± 0.09 µm/s (average ± SEM, n = 65 microtubules), which is slow compared to gliding velocities of other Unc104 motors (v = 1.2 to 2.5 µm/s; (19,21,23)). 45 out of 50 polarity-labeled microtubules were transported to the dim region, showing that NcKin3 is a plus-end directed motor (Fig. 3A).

NcKin3 gliding velocities depended on the motor concentration in the sample (Fig. 3). At saturating concentrations of more than 2 µM NcKin3 in a flow cell of approximately 3 x 18 mm², the maximum gliding velocity was 0.52 ± 0.04 µm/s (n = 179). At lower motor concentrations the gliding velocity gradually decreased to a minimum velocity of 0.30 ± 0.08 µm/s (n=74) at an NcKin3 concentration of 0.06 µM, which is significantly different from the highest density in a t-test (P < 0.0001; Fig. 3). The mean gliding velocities at the tested motor concentrations differ significantly with P < 0.0001 in an unpaired ANOVA test, and show a positive linear trend (P < 0.0001).

Below a threshold of 0.06 µM no microtubule binding was observed, even with very high microtubule concentrations. Assuming that the motors are distributed evenly on the two surfaces of the flow chamber, this corresponds to a motor density of 4.5⋅10⁹ molecules/µm². Obviously a high motor density is required to keep NcKin3 and microtubules in contact, indicating that NcKin3 is a cooperative, non-processive motor. To exclude that NcKin3 proteins clustered by streptavidin are responsible for microtubule transport at low NcKin3 concentration, residues 432 to 546 of human kinesin tail were appended to the C-terminus of NcKin3-558 (30). The resulting protein, NcKin3-558hTail, adhered unspecifically to the coverslip but behaved like the original NcKin3-558cys protein in ATPase assays. Its gliding properties were indistinguishable from the biotinylated construct.

To ensure that motor properties of NcKin3-558 proteins were not altered by the absence of the lacking 89 C-terminal wild-type amino acids we determined the gliding velocity of the full-length protein NcKin3-647. This construct turned out to adhere unspecifically to the coverslip, and displayed a gliding velocity of 0.64 ± 0.06 µm/s at saturating motor concentration, equal to the maximum gliding-velocity of the NcKin3-558
construct. Therefore, the mechanisms of full-length and truncated motors are likely to be identical.

Enzymatic properties—Processivity describes the ability of motor protein to perform several catalytic cycles on a polymeric substrate without detachment. Processive movement according to the hand-over-hand mechanism leads to distinctive kinetic features, specifically (i) an unusually high apparent binding rate in the steady-state ATPase, (ii) a large ratio between the apparent and the real binding rate measured in pre-steady state ADP release experiments and (iii) a duty ratio ≥ 50%.

To test whether NcKin3 shows these characteristics of processive motors we performed kinetic steady state and pre-steady state assays, and characterized the motility in microscopic assays.

Stoichiometry of mant-ADP release—Kinesin-1 motors show a strictly coordinated action of their two motor domains. To discern the action of the two heads of the NcKin3 dimer, mant-ADP loaded kinesin was first mixed with a 10-fold excess of microtubules, and then chased with 1 mM non-fluorescent ATP (Fig. 4). The initial fluorescence signal of the mant-ADP NcKin3 complex decreased fast upon microtubule addition, and further dropped when ATP was added. The amplitude of the second phase was essentially identical to the first phase, indicating that the ADP of one head was released upon microtubule binding, whereas the ADP of the other head was only released after ATP-binding of the kinesin microtubule complex. Importantly, the rate of the ATP-dependent fluorescence decrease was 10^3 to 10^4-fold slower (~0.02 s^{-1}) than expected for a hand-over-hand mechanism. If the gliding velocity of 0.53 µm/s of NcKin3 was caused by alternating heads that step 8 nm for each ATP, a release rate of 530 nm/(8 nm·s) = 66 s^{-1} would be expected. We examined the ADP release at microtubule concentrations ranging from 1 to 10 µM at an NcKin3 concentration of 0.9 µM (Fig. 4) to decide whether the second rate describes a microtubule dependent process. The rate of the second phase was unaffected by the microtubule concentration, as expected for a motility model where release and microtubule re-binding occurs. In the presence of AMP-PNP, a non-hydrolysable ATP analogue, the rate of the second ADP release also remained unaffected (data not shown), indicating that neither detachment of the motor from the microtubule is necessary for the second mant-ADP release, nor ATP hydrolysis. In a control experiment we analyzed the ADP release of the monomeric NcKin3-434. Here, ADP release occurred in a single-step upon addition of microtubules (Fig. 4). Addition of ATP had no further effect on the fluorescence signal, excluding the existence of a secondary ATP binding site in the NcKin3 motor head. The rate of ADP release from the monomer was faster than can be resolved manually in the spectrofluorimeter (the fastest rates we are able to measure manually are approximately 0.1 - 0.25 s^{-1}). Therefore, it is faster than the slow rate of the second head of dimeric NcKin3.

As a further control, a construct containing NcKin3 motor heads (amino acids 1-434) and a partial human kinesin tail coiled coil (termed NcKin3-434hTail) was tested. Although this construct is dimeric (Fig. S1), it behaved like the NcKin3-434 monomer in mant-ADP release assays, and lost its ADP ligand in a single, fast step. This observation implies that the dimerization domain of the NcKin3 wild-type protein, located between residues 435 and 513, confers the characteristic ADP release feature of the second NcKin3 head.

To exclude that the two-step ADP release behavior was due to properties of the methylanthraniloyl derivative of ADP, the stoichiometry of released [α-32P]ADP was determined (Supplemental data). The isolated kinesin([α-32P]ADP) microtubule complex contained 0.84 ADP ligands per NcKin3-558 dimer, indicating the release of one ADP per motor dimer (Fig. S2). The same experiment performed in the presence of ATP, or without ATP but with the monomeric NcKin434 protein, resulted in the absence of radioactive ADP in the kinesin microtubule complex, indicating the complete release of all kinesin-bound ADP. These data are consistent with the observations in mant-ADP release assays, and suggest that the mant-ADP release mimics the ADP release behavior.
In summary, these data indicate that NcKin3 contains one head with microtubule-stimulated ATPase activity, and another one with an activity that is unaffected by microtubules. This means that the ADP release from NcKin3-558 is an asymmetric two-step process and involves two heads of NcKin3 that possess different affinities for ADP. Binding of head 1 to microtubules induces ADP release from the low affinity site rapidly. The second head binds ADP tightly and turns over ATP independent from microtubule interactions at a low basal level. This observation is incompatible with a hand-over-hand mechanism for movement of NcKin3. To support these implications we compared the microtubule-induced mant-ADP release with the steady state ATPase activity, and estimated the degree of biochemical processivity (46).

Steady state ATPase kinetics—NcKin3 has an ATPase activity that is accelerated from 0.012 ± 0.001·s⁻¹ per motor head (n = 3 independent preparations) without microtubules, to 11.6 ± 4.0 s⁻¹ (n = 5) under saturating microtubule concentrations (Table 1, Fig. 5). This ATP turnover number (kcat) was calculated assuming that both NcKin3 heads contribute equally to the total microtubule-activated ATP turnover. If—as indicated by the above mant-ADP release experiments—one head cannot be stimulated by microtubules, this value has to be corrected by a factor of 2, leading to a kcat of 23.2 s⁻¹ per active head. We note that this number is still small in comparison to the gliding velocity observed in multi-motor assays. From a structural point of view, a reasonable estimate for the stroke size is 6-10 nm at maximum, which would lead to a velocity of 0.14-0.23 µm/s at 23.2 ATP·s⁻¹ (observed ~0.6 µm/s) for a processive motor (47).

The microtubule concentration required for half-maximal activation (K0.5,MT) was 1.0 ± 1.2 µM (n = 4), the ATP concentration for half-maximal ATPase activity (K0.5,ATP) was 4.0 ± 0.9 µM (n = 2). Accordingly, the ratio between kcat and K0.5,ATP, the kb,ATP value, which reflects the apparent binding rate between NcKin3 and microtubule, is 11.6 µM⁻¹s⁻¹ (23.2 if only one head contributes). This constant contains implicit information on the processivity of the enzyme (3). An enzyme that performs several catalytic cycles upon a single encounter with a substrate will show a kcat/K0.5,MT that is much larger (typically much more than 100 µM⁻¹s⁻¹) than the actual bimolecular binding rate that can be measured in pre-steady state assays.

To compare apparent and actual bimolecular NcKin3 microtubule binding rate, we measured the transient rate of ADP release in a stopped-flow experiment (Fig. 5). Mant-ADP was used to monitor the dissociation of ADP from NcKin3 upon binding to the microtubule. At saturating microtubule concentrations, ADP release of NcKin3 occurred at a rate of kmax(ADPrelease)= 7.1 ± 2.0 s⁻¹ with a K0.5,MT of 0.94 ± 0.04 µM (n = 3) (Fig. 5). These values are comparable to kcat and K0.5,MT measured in steady-state assays, suggesting that (as for other kinesins) ADP release is rate limiting in NcKin3’s catalytic cycle. From these parameters, the physical bimolecular rate, kb,ADP, can be calculated as kb,ADP = kmax(ADPrelease)/K0.5,MT = 7.55 µM⁻¹s⁻¹. This rate constant is 1.5 or 2.9-fold larger than the apparent rate constant determined in steady state assays, and the ratio kb,ratio = kb(ADP)/kb(ATP) = 1.5 or 2.9, depending on the model. According to (3), this number is an estimate for the numbers of ATP molecules hydrolyzed per encounter between NcKin3 and the microtubule, indicating that NcKin3 is a motor protein that performs one or few catalytic cycle per microtubule encounter.

Microtubule detachment—To test whether the release rate of NcKin3 from microtubules agrees with a low degree of processivity, we measured the ATP-induced dissociation of the NcKin3-558 microtubule complex (Fig. 6). A kinesin that detaches after a single step or chemical cycle is expected to show a detachment rate as fast as its kcat, or faster.

We measured detachment rates in a stopped-flow apparatus, where the dissociation of kinesin and microtubules is observed by the change of light scattering. As this assay is only interpretable if the weak microtubule-binding nucleotide state is known, we tested the affinities of monomeric and dimeric NcKin3 constructs in the presence of ADP and the non-hydrolysable ATP analogue AMP-PNP in microtubule co-sedimentation assays (Fig. S4). As observed for all other kinesins tested so far, both mutants display a roughly 7-fold lower K0 in the ADP-state compared to the AMP-PNP state (0.20 ± 0.16 µM), suggesting that NcKin3...
dissociates from the filament in the ADP-bound state (1.4 ± 0.8 µM).

Microtubule detachment rates were measured in dependence of ATP concentrations from 0-64 µM. The light scattering signals showed a single exponential time course. The absence of a scattering signal change in control experiments without microtubules and without ATP showed that the traces represented dissociation events. The rates showed a hyperbolic dependence on the ATP concentration with a maximum detachment rate of \( k_{\text{max}} = 22.19 \pm 7.64 \text{ s}^{-1} \) and a half-maximal activation constant of \( K_{1/2} = 2.22 \pm 2.75 \mu\text{M} \) ATP (Fig. 6). Thus, \( k_{\text{max}} \) was similar to the steady state turnover rate (\( k_{\text{cat}} \sim 23 \text{ s}^{-1} \)), the \( K_{1/2} \) similar to the \( K_{\text{M,ATP}} \) value of steady-state ATP hydrolysis (\( K_{\text{M,ATP}} = 4.0 \pm 0.9 \mu\text{M} \)).

To rule out that the observed scattering signal results from mixing artifacts in stopped-flow experiments detachment rates were also measured in a flash-photolysis apparatus. Here, light-induced liberation of ATP from caged ATP induces the dissociation of the NcKin3 microtubule-complex (Fig. S3). These assays showed single-exponential release kinetics, excluding the existence of fast rates invisible in stopped-flow assays. They also support that the traces observed in stopped-flow scattering assays represent dissociation events. The slower rates observed in flash photolysis experiments were most likely due to the inhibitory effect of caged ATP (48).

**Duty ratio** - To support the hypothesis that NcKin3 is a dimeric, non-processive motor, we estimated its duty ratio from comparisons of \( K_{0.5,\text{ATP}} \) in gliding and enzymatic assays (Fig. 7) (47). The duty ratio, \( r \), is defined as the time fraction of the catalytic cycle a motor spends in the strongly filament-bound state. The ratio of \( K_{0.5,\text{ATP}} \) measured in the steady-state ATPase assay and \( K_{0.5,\text{ATP}} \) measured in the multiple motor gliding assays is a reasonable estimate for this value because the \( K_{0.5,\text{ATP}} \) in the gliding assay reflects the situation where half of the molecules ‘wait’ for ATP in the microtubule-bound state, whereas the \( K_{0.5,\text{ATP}} \) in the steady-state ATPase results from intermediates of the entire cycle, including the un-attached states. (47). For NcKin3 the values for \( K_{0.5,\text{ATP}} \) in gliding and ATPase assays strongly deviate. In the steady-state ATPase we measured \( K_{0.5,\text{ATP}} = 4.0 \pm 0.9 \mu\text{M} \), in the multiple motor gliding assays \( K_{0.5,\text{ATP}} = 138.0 \pm 49.5 \mu\text{M} \) (Fig. 6). This results in a duty ratio of \( r = 0.03 \) which means that NcKin3 spends only three percent of the catalytic cycle attached to the filament. This ratio is typical for non-processive enzymes that work in ensembles. Motors that move processively based on a hand-over-hand mechanism of two motor heads require duty ratios of at least 0.5.

Knowledge of the duty ratio, \( k_{\text{cat}} \) and gliding velocity (\( v_{\text{glid}} \)) allows estimation of working distances (\( \Delta \)) of molecular motors (47):

\[
\Delta = \frac{(v_{\text{glid}} \cdot r)}{k_{\text{cat}}}
\]

Accordingly, NcKin3-558 is calculated to have a working distance of \((0.59 \mu\text{m s}^{-1} \cdot 0.03) / 23.2 \text{ s}^{-1} = 0.8 \text{ nm}\) (Fig. 8). In comparison to processive kinesins, or the distance between two tubulin dimers in the microtubule (8.1 nm), this is a very short distance, and hardly compatible with a hand-over-hand mechanism.

**Discussion**

Our data indicate that NcKin3 is a dimeric and non-processive kinesin motor with unique mechanistic properties (Fig. 8): (1) The velocity for microtubule gliding depends on the NcKin3 coating density of the flow chamber, and is faster than predicted from ATP turnover and reasonable stroke size dimensions. This indicates a cooperative action of several motors at a single microtubule. (2) The ADP release from the two heads is not coupled and only one head interacts with the microtubule. Microtubule-induced mant-ADP release is a two-step process with a fast and a slow rate. The slow rate, presumably resulting from the free, microtubule unbound head, is two orders of magnitude slower than the \( k_{\text{cat}} \) and independent of the microtubule concentration. This excludes a hand-over-hand mechanism. (3) The chemical processivity of NcKin3 is low. The ratio of apparent bimolecular binding in steady state ATPase assays and mant-ADP release experiments of 1.5 to 3 suggests only few catalytic cycles per microtubule encounter, after which NcKin3 detaches from the microtubule. This is supported by microtubule detachment rates that resemble the \( k_{\text{cat}} \). 4. NcKin3 has a low duty ratio (\( \sim 0.03 \),
implying that it cannot use a hand-over-hand type mechanism. If the motor is bound only 3\% of its chemical cycle time to the filament, even a dimer is not sufficient to support a continuous microtubule association. These arguments suggest that NcKin3 also acts cooperatively in the cellular environment. In vivo, the protein is found on mitochondria, which are likely to harbor a large number of motors. Coating density fluctuations or local clustering might be a means of regulating mitochondria shape and transport speed.

The comparison of NcKin3 with other kinesin motors shows important features that have not been observed in other kinesins so far. As our data are incompatible with a hand-over-hand mechanism, NcKin3 it clearly differs from processive Kinesin-1, -2 and -5 motors. NcKin3 also differs from other Kinesin-3 motors, although some of its kinetic features may hold true for other Kinesin-3 members as well. KIF1A has been extensively used as a model for Kinesin-3 motors. It can function as a monomeric, quasi-processive motor (19,45). In this motility mode, it presumably proceeds by a combination of ATP-dependent microtubule affinity changes, and the action of the K-loop that tethers the motor at the microtubule during phases of weak affinity (18,20). It was shown that the average displacement was coupled to a single ATP hydrolysis, although fluctuations in the velocity and directionality indicate a stochastic, diffusive nature of movement. An asymmetric binding potential might impose the bias to the microtubule plus end. In contrast to KIF1A, NcKin3 does not have the structural prerequisites to move in this manner because it does not contain a fully developed K-loop (Fig. 1).

Another member of the Kinesin-3 family has been studied in detail, the Unc104 motor from C. elegans. The bacterially expressed CeUnc104 motor is monomeric and does not support processive movement in single molecule assays (49). Only CeUnc104 mutants that were dimerized artificially by a synthetic coiled coil domain showed processive runs (23). Transport of associated cargo was only observed when two protein chains were artificially connected or forced to dimerize by very high local motor concentrations (24). It is unclear whether the same mechanism applies to KIF1A because the constructs used for the studies are not comparable.

Mouse KIF1C and D. discoideum Unc104, however, were found as a dimers in vivo, leaving the possibility that KIF1A behaves aberrantly (44,50).

The observations on CeUnc104 indicate that Kinesin-3 activity might be regulated by dimerization in vivo. This is clearly not the case for NcKin3 that is constitutively dimeric but not processive, excluding that regulation by dimerization is a general feature of Kinesin-3 motors. Whether the mechanistic basis of dimerized CeUnc104 constructs differs fundamentally from NcKin3 is unclear because CeUnc104 has not been investigated in biochemical assays for processivity. It may be interesting to investigate other Kinesin-3 motors in kinetic assays and to test their head-head coordination.

In its kinetic properties, NcKin3 most closely resembles Ncd, a non-processive minus-end directed Kinesin-14 from D. melanogaster (9-13,51-54). Like NcKin3, Ncd consists of two identical polypeptide chains that show different affinities to ADP. However, unlike NcKin3 both Ncd motor domains are able to interact with the microtubule (11,55,56). First, the motor head with the lower affinity for ADP binds to the microtubule while the second head is detached and points towards the microtubule minus-end (15,51). According to (13), the motor detaches from the microtubule after binding a new ATP, and is then competent to re-bind with either of its heads. Alternatively, ATP hydrolysis of the first head may allow binding of the partner head to the next β-tubulin subunit in the minus end direction (52). Subsequently, the other head would hydrolyze a second ATP before it entered a state where the motor dissociated from the microtubule. According to this model, one step of Ncd requires the hydrolysis of 2 ATP molecules.

Either mechanism is unlikely for NcKin3 because the mant-ADP release from the high-affinity site was much slower than $k_{cat}$ and equal to the basal activity. It was also independent of the microtubule concentration up to a tenfold excess of tubulin over motor heads. Hence, there is no sign of microtubule interaction of the second head. However, like Ncd NcKin3 probably uses a power-stroke mechanism for generation of motility.
(15,55), but since Ncd is a minus-end motor, the stroke has to be oriented towards the opposite direction.

In summary, these comparisons show that NcKin3 moves by a mechanism so far unknown for kinesins. Possibly, the motor is adapted to special requirements of mitochondria shaping. A high local motor accumulation may allow microtubule interaction and local expansion of the mitochondrion. Filamentous fungi are known to possess networks of elongated mitochondria, whose shape depends on intact microtubules (57-59). Alternatively, the motor serves additional cellular functions, which require a non-processive motor.

An interesting future question is the functional role of the second motor head. The truncated constructs used in this study provide only limited insight because the lack of large protein portions is likely to induce major structural defects. More sophisticated chimeric constructs are required to overcome these limitations. Possibly, one of the two heads contacts the microtubule in a similar way as the K-loop of Kif1A and acts as a passive tether. Alternatively, the second head might influence the catalytic cycle of the microtubule-bound head by an allosteric effect. It will be interesting to study artificial single-headed heterodimeric motors to elucidate the function of the second head. This and other studies show that the hand-over-hand mechanism may not be a general principle for kinesin motors. It will therefore be interesting to track down the elementary process of generation of motility.

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Figure Legends

Fig. 1. Domain architecture of NcKin3

(A) The upper part of the scheme shows the domain organization of NcKin3 and a sequence comparison of regions of specific interest. NcKin3 has a subgroup-specific N-terminal extension of 36 amino acids (absent in mouse KIF1A, C. elegans Unc104 and D. discoideum Unc104), followed by the conserved core motor domain. The motor domain contains an unusual K-loop with fewer lysines than in other Kinesin-3 motors, interspersed with Ser, Gly and Pro residues. A short stretch corresponding to the neck-linker of Kinesin-1 motors follows before a presumable coiled coil region starts (for comparison the human kinesin (HsKIF5B) neck coiled coil is boxed in the sequence alignment (60)). The predicted coil phase is indicated by letters a-g above the sequence and clearly dissimilar to the human kinesin coiled coil. The C-terminal 134 residues (tail) do not show any recognizable sequence motifs and are highly Asp-rich between position 558 and 647. (B) The SDS-gel below the scheme shows bacterially expressed, purified truncation mutants used in the study (the number indicates the last NcKin3 residue).

Fig. 2. Dimerization of NcKin3

The upper part of the figure shows example elution profiles of truncated NcKin3 mutants on a gel filtration column (left) and a SDS gel of a sucrose density gradient centrifugation (right). The quantification (Experimental procedures) resulted in Stokes radii and sedimentation coefficients summarized in the table (lower part), and served to calculate the oligomerization state of the constructs. Only proteins containing more than two thirds of the predicted coiled coil form dimers.

Fig. 3. Gliding velocity of NcKin3 in multi-motor assays

Panel (A) shows polarity-labeled microtubules in a gliding assay of NcKin3. The bright end (microtubule minus-end) is leading, indicating that NcKin3 is a microtubule plus-end motor. Panel (B) shows the dependence of the gliding velocity on the surface density of motors. At least 50 microtubules were analyzed (grey dots) and averaged (red dots; error bars show SEM). At lower densities NcKin3 becomes significantly slower, indicating cooperativity between motors.

Fig. 4. Stoichiometry of microtubule-activated mant-ADP release from NcKin3-558 and -434

Mant-ADP release from NcKin3-558 (black symbols) occurs in two steps that cause together a 30% decrease of the fluorescence signal. Mant-ADP-loaded NcKin3-558 (~0.9 µM) was mixed with a 10-fold excess of microtubule (10 µM) in low salt 12A25+ buffer to ensure complete
binding of kinesin to the filament. The signal rapidly reaches a plateau at 0.85 relative fluorescence units, indicating a 50% release of mant-ADP. Addition of 1 mM ATP causes the signal to decrease by another 50% to 0.7 relative fluorescence units, the fluorescence level of free mant-ADP in solution. The second phase of the signal obeys the time course of a single exponential function (grey dotted line). The rate of the second phase (inset) does not depend on the microtubule concentration. Likewise the presence of the non-hydrolysable ATP analogue AMP-PNP (1 mM) instead of ATP, which inhibits detachment from the MT-bound head, does not change the signal of the second ADP release (data not shown). The monomeric variant NcKin3-434 (grey) loses mant-ADP in a single step upon addition of microtubules.

Fig. 5. Chemical processivity of the NcKin3 protein.

(A) The steady state ATPase rate of NcKin3 was measured at variable microtubule concentrations. Based on the concentrations of polypeptide chains the $k_{\text{cat}}$ was 11.6 s⁻¹. Considering that only one of two heads participates in the microtubule-dependent ATPase reaction the $k_{\text{cat}}$ is 23.2 s⁻¹. The $K_{0.5,\text{MT}}$ was 1.0 µM, $k_{\text{cat}}/K_{0.5,\text{MT}}$ was 11.6 or 23.2 µM⁻¹s⁻¹.

(B) To determine the ADP release rate mant-ADP loaded NcKin3 was mixed with microtubules in a stopped-flow apparatus. The rates of the single-exponential decays were plotted against the microtubule concentration. Each data point is an average of at least five individual stopped-flow traces. The data points were fitted to a hyperbolic function (solid line), which was the best fit for the given data. The $k_{\text{obs}}$ was 7.55 s⁻¹, $K_{0.5,\text{MT}}$ was 0.94 µM, $k_{\text{obs}}/K_{0.5,\text{MT}}$ was 8.03 µM⁻¹s⁻¹. The ratio between the steady state and the pre-steady state binding rates ($k_{\text{bi}}$ ratio, (46)) is thus 1.5 or 2.9.

Fig. 6. Microtubule detachment of Nckin3

The dissociation of the pre-formed NcKin3 microtubule complex was induced by ATP in a stopped-flow apparatus and followed by the change of the light scattering signal. Panel A shows a representative average from 5 traces. The grey curve is a mono-exponential fit to the data that was used to derive the rates, $k_{\text{obs}}$. Panel B plots $k_{\text{obs}}$ against the ATP concentration, and shows a hyperbolic dependence with a $k_{\text{max}}$ of 22.2 s⁻¹ and a $K_{1/2}$ of 2.2 µM, comparable to the steady state parameters $k_{\text{cat}}$ and $K_{0.5,\text{ATP}}$.

Fig. 7. Duty ratio

Panels A and B show example measurements of the gliding velocity and the ATPase rate of NcKin3-558 in dependence of the ATP concentration. The ratio of $K_{\text{M,ATP}}$ in these assays served to estimate the duty ratio (47). The average $K_{\text{M,ATP}}$ in ATPase assays was 4.0 ± 0.9 µM and 138.0 ± 49.5 µM in gliding assays, suggesting a duty ratio of 0.03.
Fig 8. Model of NcKin3 function

NcKin3 is a homodimeric motor (blue) that interacts with one head with the microtubule (red), whereas the second head (white) is not activated by microtubules. ATP-binding to the nucleotide-free, microtubule-bound head and subsequent catalytic events induce a power-stroke that leads to a working distance of a calculated size of 0.8 nm (see text, (47)). Afterwards, the motor dissociates from the microtubule (probably in the ADP-state) and has to be converted into the pre-power-stroke conformation to allow the next catalytic round.
**Tables**

**Table 1: Kinetic properties of NcKin3-558**

| Parameter                                                                 | Value                  |
|---------------------------------------------------------------------------|------------------------|
| $k_{basal}$ (basal ATPase activity)                                        | $1.2 \times 10^{-3} \text{ s}^{-1}$ |
| $k_{cat}$ (microtubule-activated ATPase activity)                          | two / one active head per dimer / $11.6 / 23.2 \text{ s}^{-1}$ |
| $K_{0.5,MT}$ (microtubule concentration for half-maximal ATPase activity) | $1.0 \mu M$            |
| $K_{0.5,ATP}$ (ATP concentration for half-maximal ATPase activity)         | $4 \mu M$              |
| $k_{bi,ATP}$ (bimolecular binding rate between NcKin3 and microtubule)    | $11.6$ or $23.2 \mu M^{-1}\text{s}^{-1}$ |
| $k_{obs}$ (ADP release)                                                   | $7.55 \text{ s}^{-1}$  |
| $K_{0.5,MT}$ (ADP release)                                                | $0.94 \mu M$           |
| $k_{bi,ADP}$ (physical binding rate between NcKin3 and microtubule)       | $8.03 \mu M^{-1}\text{s}^{-1}$ |
| $k_{bi,ratio}$ ($k_{bi,ATP}$/$k_{bi,ADP}$)                               | $1.5$ or $2.9$         |
Figures

**Fig. 1**

### A

| N-terminal extension | Motor Domain | Coiled-coil | Tail |
|----------------------|--------------|-------------|------|
| MmKif1A              | 37           | 415 434     | 513 558 647 |
| CeUnc104             |              |             |      |
| DdUnc104             |              |             |      |
| NcKin3               | MIPSNLDGVRQ QTRSNVTSP LRPRDDTASS FVSXDPGANV RVVVR... |

**K-loop**

- MmKif1A: LAEMSGFNLKQKKTQ-
- CeUnc104: LAAEST-
- DdUnc104: LAENST-
- NcKin3: LADPKSSASRFSPVTNSQGRTPFGLVSFFVFPY...

### B

- 66 kDa
- 45 kDa
Fig. 2

C

| Construct       | $r_{\text{Stokes}}$ [nm] | $S_{\text{w,20}}$ [s] | Deduced MW [kDa] | Calculated MW [kDa] | Oligomerization state |
|-----------------|--------------------------|------------------------|-------------------|----------------------|----------------------|
| NcKin3-415      | 3.25 ± 0.03              | 3.9 ± 0.2              | 52724.9           | 47565                | monomer              |
| NcKin3-434      | 3.47 ± 0.01              | 3.9 ± 0.3              | 55912.0           | 49029                | monomer              |
| NcKin3-457      | 3.79 ± 0.06              | 4.1 ± 0.2              | 63441.0           | 51963                | monomer              |
| NcKin3-470      | 3.97 ± 0.03              | 3.6 ± 0.3              | 59243.2           | 53452                | monomer              |
| NcKin3-488      | 3.56 ± 0.30              | 4.4 ± 0.2              | 65157.2           | 55478                | monomer              |
| NcKin3-513      | 5.34 ± 0.20              | 4.9 ± 0.3              | 106921.1          | 58503                | dimer                |
| NcKin3-558      | 4.99 ± 0.10              | 5.4 ± 0.4              | 110615.1          | 63081                | dimer                |
Fig. 3
Fig. 4

[Diagram showing fluorescence relative to time with conditions for + Microtubules and + ATP, and inset showing k(ADP release) vs. microtubule concentration.]
**Fig. 5**

A

![Graph A](image1)

B

![Graph B](image2)

**k (ATP hydrolysis), s⁻¹ vs. microtubule concentration, μM**

**k_{obs}, s⁻¹ vs. microtubule concentration, μM**

Inset:

![Fluorescence intensity vs. time](image3)
**Fig. 7**

**A**

Graph showing gliding velocity (μm/s) as a function of ATP concentration (mM). The graph displays a sigmoidal curve with data points and a fitted line.

**B**

Graph showing specific ATPase activity (k, s⁻¹) as a function of ATP concentration (μM). The graph also displays a sigmoidal curve with data points and a fitted line.
Fig. 8
Kinetic and mechanistic basis of the non-processive Kinesin-3 motor NcKin3
Sarah Adio, Marieke Bloemink, Michaela Hartel, Sven Leier, Mike A. Geeves and Guenther Woehlke

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