Molecular Cloning and Functional Studies of *Neurospora crassa* KIF1, a New Member of the UNC-104/KIF1 Family of Kinesin-Like Proteins

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CONTENTS

ABBREVIATIONS:

A	Absorption
aa	Amino acid
ATP	Adenosine-5'–trisphosphate
ACES	N-[2-Acetamido]-2-aminoethanesulfonic acid
AMP-PNP	Adenosine-5'–[β, γ-imido]-triphosphate
approx.	Approximately
BCIP	Bromo-chloro-indolyl phosphate
bp	Base pairs
BSA	Bovine serum albumin
cDNA	Complementary DNA
Ce	*Caenorrhabditis elegans*
CIP	Calf intestinal phosphatase
cpm	counts per minute
Da	Dalton
Dd	*Dictyostelium discoideum*
dCTP	2'-Deoxycytidine 5'-triphosphate
dH_2O	Distilled water
dist.	Distilled
DMSO	Dimethylsulfoxide
DNA	Desoxyribonucleic acid
dNTP	Desoxyribonucleotide trisphosphate
DTT	Dithiothreitol
E. coli	*Escherichia coli*
EDTA	Ethylene-diamine-tetraacetic acid
EGTA	Ethyleneglycol-bis(2-aminoethylether)-N,N'-tetraacetic acid
et al.	And others
FGSC	Fungal Genetics Stock Center
Fig.	Figure
FPLC	Fast Performance Liquid Chromatography
GTP	Guanosin-5'-triphosphate
h	Hour
Hs	*Homo sapiens*

IPTG	Isopropyl-β-D-thiogalactopyranoside
k	Kilo
KIF	Kinesin family protein
Kin	Kinesin
l	Liter
M	Mol/l
min	Minute
μm	Micrometer
Mm	*Mus musculus*
MOPS	Morpholinopropanesulfonic acid
Mt	Microtubules
n. d.	not determined
NADH	Nicotine adenine dinucleotide
NBT	Nitroblue-tetrazolium chloride
Nc	*Neurospora crassa*
NEM	N-Ethylmaleimide
nm	Nanometer
OD	Optical density
p.	Page
PAA	Polyacrylamide
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
PEM	N-[2-(1-Piperazinyl)ethyl]maleimide
pfu	Plaque forming units
PIPES	Piperazine-N,N'-bis-[2-ethanesulfonic acid]
Rn	*Rattus norvegicus*
rpm	Revolutions per minute
r_{Stokes}	Stokes radius
SDS	Sodium dodecyl sulfate
sec	Second
$S_{w,20}$	Svedberg constant
TAME	Nα-p-tosyl-l-arginine-methylester
TEMED	N,N,N',N'-tetramethylethylenediamine
Tris	Tris-hydroxymethyl-ammoniumethane

Triton X-100	t-Octylphenoxypolyethoxyethanol
Tween 20	Polyoxyethylene-sorbitanemonolaureate
U	Units
Um	*Ustilago maydis*
V	Volt
v/v	Volume per volume
w/v	Weight per volume
WT	Wild-type

1. INTRODUCTION

Kinesins are molecular motor proteins that hydrolyze ATP to transport cargo, e.g. vesicles or organelles, along microtubules in the cell. They are also essential for chromosome alignment, spindle assembly and elongation (Hirokawa, 1998; Endow, 1999). The kinesins form a superfamily with over 100 proteins that can be subdivided into at least 10 subfamilies (Hirokawa, 1998; Kim and Endow, 2000). Most kinesins move their cargo towards the plus-ends of microtubules.

Kinesin family members share a conserved motor domain that comprises approximately 350 amino acid residues. The motor domain contains the binding sites for microtubules and ATP. According to the location of the motor domain they are classified into N-kinesins, M-kinesins and C-kinesins. The N-kinesins have their motor domain at the NH_2-terminus, the M-kinesins in the middle and the C-kinesins at their COOH-terminus. The majority of the kinesins belong to the N-type (Hirokawa, 1998; Miki et al., 2001).

Most kinesins are multimeric proteins. Conventional kinesin, for example, is composed of two heavy chains, which dimerize, and two light chains. The heavy chain contains the highly conserved motor domain and the tail domain, which is thought to be responsible for cargo binding.

The work presented here focuses on a new member of the UNC-104/KIF1 family of kinesin-like proteins, NcKIF1 from the ascomycete *Neurospora crassa*. The UNC-104/KIF1 family forms a subgroup of the kinesin superfamily (Table 1). The members of this family are known to be involved in the transport of membranous organelles towards the plus-ends of microtubules (Hirokawa, 1998; Bloom, 2001). Known functions of the proteins are:

(i) the transport of synaptic vesicles or synaptic vesicle precursors (UNC-104 from *Caenorhabditis elegans*, Hall and Hedgecock, 1991; MmKIF1A, that transports a subset of synaptic vesicle precursors and plays an important role in neuronal function and survival, Okada et al., 1995, Yonekawa et al., 1998; MmKIF1Bb (MmKIF1Bβ), Zhao et al., 2001 and HsATSV, the human orthologue of mouse KIF1A, Furlong et al., 1996)

(ii) the transport of mitochondria (MmKIF1B, Nangaku et al., 1994)

(iii) involvement in endoplasmic reticulum-Golgi transport (HsKIF1C, Dorner et al., 1998)

(iv) the transport of early endosomes (UmKin3, Wedlich-Söldner et al., 2002) and possibly,

(v) a contribution to the delivery of cerebrospinal fluid to the ventricular space (RnKIF1D, Rogers et al., 1997).

Table 1. Members of the UNC-104/KIF1 family and their functions.

Member	Calculated molecular mass of the polypeptide [kDa]	Monomer/Dimer	Functions	References
UmKin3	185	n. d.	Transport of early endosomes in the fungus *Ustilago maydis*	Wedlich-Söldner et al., 2002
DdUnc104	248	Dimer	Dominant plus-end-directed organelle transport motor in *Dictyostelium discoideum*	Pollock et al., 1999
Ceunc104	180	Monomer [1)]	Axonal transport of synaptic vesicles	Hall and Hedgecock, 1991; Pierce et al., 1999
MmKIF1A	192	Monomer	Transport of a subset of synaptic vesicle precursors, plays an important role in neuronal function and survival	Okada et al., 1995; Yonekawa et al., 1998
MmKIF1B	130	Monomer	Transport of mitochondria	Nangaku et al., 1994
MmKIF1Bb (MmKIF1Bβ)	204	n. d. [2)]	Transport of synaptic vesicle precursors	Zhao et al., 2001
RnKIF1D	122	n. d. [3)]	Assumed to have an important role in the secretory pathway responsible for delivering cerebrospinal fluid to the ventricular space	Rogers et al., 1997
HsKIF1C	123	Can form dimers	Endoplasmic reticulum-Golgi transport	Dorner et al., 1998; Dorner et al., 1999
HsATSV	191	Monomer (human orthologue of MmKIF1A)	Probably the transport of synaptic vesicles	Furlong et al., 1996

[1)] It was suggested that Ceunc104 and KIF1A may act as functional dimers in vivo (Tomishige et al., 2002).

[2)] From secondary structure prediction MmKIF1Bb may exist as a monomer (Gong et al., 1999).

[3)] According to secondary structure prediction RnKIF1D is a monomeric motor (Rogers et al., 1997).

The sizes of the known members of the UNC-104/KIF1 family range between 122 kDa and 248 kDa per polypeptide chain. Most members of this family are monomeric proteins. Known exceptions are DdUnc104, which was shown to be a dimer (Pollock et al., 1999), and human KIF1C, which can also form dimers in vivo (Dorner et al., 1999). The members of the UNC-104/KIF1 family have their motor domain at the NH_2-termini, therefore they are classified as N-type kinesins (Hirokawa, 1998).

The members of the UNC-104/KIF1 family of kinesin-like proteins have as a special feature a K-loop in their motor domain. The K-loop is an amino acid insertion in loop 12 (L12) of the motor domain. In this loop several lysines or positively charged residues can be found in sequence (Fig.4, 3.1.2.). The K-loop is surmised to work as an extra binding domain for microtubules, which dramatically increases the affinity to microtubules (Kikkawa et al, 2000). In the case of the motor molecule KIF1A, the positively charged loop 12 (K-loop) was demonstrated to interact with the glutamate-rich, highly negatively charged C-terminal region of tubulin (E-hook) (Okada and Hirokawa, 2000).

This work presents studies on NcKIF1, a new member of the UNC-104/KIF1 family of kinesin-like proteins. Previous work had identified a partial NcKIF1 sequence isolated from a mycelial cDNA library of *N. crassa* (Seiler, 1999). Two positive clones containing the NcKIF1 sequence were also found in a cosmid library (Orbach, 1994; FGSC).

The sequence of NcKIF1 was isolated again from a *N. crassa* cDNA library. A short construct of NcKIF1 containing the conserved motor domain and the following 14 amino acids was generated to study the basic properties of this motor. The full-length protein was also investigated. The aim of this work was a characterization of the basic biochemical properties such as ATPase activity and gliding velocity, multimerization, and expression in mycelia of *N. crassa*.

2. MATERIALS AND METHODS

2.1. Materials

Unless otherwise indicated, chemicals were obtained from Sigma-Aldrich (Deisenhofen), Merck (Darmstadt), Carl Roth (Karlsruhe), Serva (Heidelberg), Fluka (Buchs), BIO-RAD (Munich) and Roche Diagnostics (Mannheim) and were of p. a. quality. All restriction enzymes and buffers as well as other DNA-modifying enzymes were purchased from New England Biolabs (Frankfurt am Main). For the PCR-reactions the Expand High Fidelity PCR-System (Roche Diagnostics, Mannheim) was used, as not otherwise indicated. Antibodies were obtained from Sigma (Deisenhofen). Oligonucleotides were provided by Gibco-BRL-Lifetech (Karlsruhe).

2.2. Vectors and strains

2.2.1. Vectors

As vectors were used: pT7-7 (Tabor, 1990) and pBluescript SK- (Stratagene). Furthermore, pT12-1 (Henningsen U., 1997), λ10, λ11/2, X2G12 and G2B8 (Seiler S., 1999) were used.

2.2.2. Bacterial strains

The following *Escherichia coli* strains were used: XL1-Blue (Stratagene) and DH5α (Sambrook et al., 1989) for cloning; BL 21 (Studier et al., 1990), BL 21 RIL (Stratagene) and MC1061 (New England Biolabs) for protein expression; XL1-Blue MRF‘ (Stratagene) and SOLR (Stratagene) for screening of cDNA and in vivo excision.

2.2.3. *N. crassa* strains

Neurospora crassa WT 74 A and NcKin null mutant constructed from WT 74 A (Seiler et al., 1997) were used.

2.3. Cultivation of *E. coli*

Bacteria were cultivated according to standard methods (Sambrook et al., 1989) on agar plates or in liquid culture (240 rpm). The cultivation temperature was 37°C. For protein expression a

temperature of 22°C was used. For long-term storage, 500 µl of bacterial culture were mixed with an equal amount of 60 % glycerol and stored at -70°C.

Media (all from Sambrook et al., 1989)

LB-medium

10 g/l tryptone, 5 g/l yeast extract, 10 g/l NaCl, pH 7.0.

LB-amp-medium

LB medium with 100 µg/ml ampicillin.

LB-amp-chloramphenicol-medium

LB-medium with 100 µg/ml ampicillin and 25 µg/ml chloramphenicol.

SOB-medium

20 g/l tryptone, 5 g/l yeast extract, 10 mM NaCl, 2.55 mM KCl, 10 mM $MgCl_2$.

SOC-medium

SOB-medium supplemented with 10 mM $MgSO_4$ and 20 mM glucose.

Agar plates

1.5 % agar in LB.

2.4. Cultivation of *Neurospora crassa*

2.4.1. Growing and storage of conidia

An Erlenmeyer flask containing minimal medium agar was inoculated with conidia from a conidial stock of *Neurospora crassa*. The flask was covered with an autoclaved foam rubber pad. The culture was grown for 14 days at 25°C under light. After that time the conidia were harvested by washing the agar with autoclaved distilled water and stored at 4°C. For a conidial stock the agar was washed with autoclaved distilled water containing 10 % milk powder (Roth). 200 µl of this suspension was mixed with silica gel (Silica gel 60 (0.2 - 0.5 mm) for column chromatography, Merck), dried in an exsiccator and stored at 4°C.

2.4.2. Culture and media

For growth of *N. crassa* mycelium minimal medium was inoculated with conidia and shaken for 16 h at 25°C under light. For the crude extract a sterile culture flask containing 4 l of

minimal medium inoculated with conidia was also used. This culture was grown for 16 h at 25°C under light as well and constant aeration (Sebald et al., 1979). Then the cells were harvested and their weight determined.

Media

Vogels-medium (50x)

15 % (w/v) sodium citrate x $2H_2O$, 25 % (w/v) KH_2PO_4, 10 % (w/v) NH_4NO_3, 1 % (w/v) $MgSO_4$ x 7 H_2O, 5 % (w/v) $CaCl_2$, 5 % (v/v) trace element solution, 2.5 % (v/v) biotin solution, H_2O dist.

Trace element solution

5 % (w/v) citric acid, 5 % (w/v) $ZnSO_4$ x 7 H_2O, 1 % (w/v) $Fe(NH_4)_2(SO_4)_2$, 0.25 % (w/v) $CuSO_4$, 0.05 % (w/v) $MnSO_4$ x H_2O, 0.05 % (w/v) H_3BO_3, 0.05 % (w/v) Na_2MoO_4 x $2H_2O$, H_2O dist.

Sucrose solution

50 % (w/v) sucrose, H_2O dist.

Minimal medium

2 % (v/v) Vogels-medium, 4 % (v/v) sucrose solution, H_2O dist.

Minimal medium agar

1.5 % of agar in minimal medium

2.5. Molecular biology methods

2.5.1. Agarose gel electrophoresis

The separation of DNA fragments according to their size was performed using gels with 1 % agarose in TAE buffer. For the detection of DNA fragments 0.05 µg/ml ethidium bromide was added to the liquid agarose. Gels were run with 50 - 80 V. Before loading the samples were mixed with 1/5 volume of 6 x TAE loading dye. Bands were detected by UV-illumination and documented with the Eagle Eye II system (Stratagene, Heidelberg).

50 x TAE: 2 M Tris·HCl, pH 7.4, 0.57 % acetic acid, 50 mM EDTA, pH 8.0

6 x DNA loading dye: 30 % glycerol, 0.25 % bromophenol blue, 0.25 % xylene cyanol

2.5.2. DNA extraction from agarose gels

Bands were excised, transferred to sterile Eppendorf cups, weighed and purified with the Qiaquick spin columns (Qiagen) following the instructions of the manufacturer.

2.5.3. Determination of DNA and RNA concentration

DNA and RNA concentration in solutions was determined by measuring the A_{260} with a spectrophotometer. An A_{260} of 1.0 corresponds to 50 μg/ml of DNA and to 40 μg/ml of RNA (Sambrook et al., 1989).

2.5.4. Preparation of plasmid DNA

Plasmid DNA was prepared from an overnight culture using the Qiagen Plasmid Midi kit (Qiagen, Hilden) according to the manufacturer's manual. The DNA could then be used for restriction digests, PCR, sequencing and transformation of bacteria.

2.5.4.1. Analytical preparation of plasmid DNA

3 ml LB-amp cultures were grown overnight at 37°C, 240 rpm. 1.5 ml of each culture was then transferred into an Eppendorf cup and centrifuged in a tabletop centrifuge at maximum speed for 30 sec. The supernatant was discarded and the pellet resuspended in 100 μl lysis buffer (10 mM Tris·HCl, pH 8.0, 1 mM EDTA, 15 % (w/v) sucrose, 2 mg/ml lysozyme, 0.2 mg/ml RNase A (from bovine pancreas, DNase free), 0.1 mg/ml BSA). The cups were shaken for 5 min, then heated for 60 sec at 95°C and immediately put on ice for 60 sec. The samples were centifuged for 15 - 20 min in a tabletop centrifuge at maximum speed. The supernatant containing the plasmid was used for restriction analysis (Berghammer et al., 1993). The remaining culture was taken for the inoculation of a larger culture for a plasmid preparation according to the Qiagen Plasmid Midi kit protocol, see 2.5.4. in a positive case.

2.5.5. DNA cleavage with restriction enzymes

Restriction digests were performed using the buffer system and temperature recommended by the manufacturer. Incubation time was at least 1 h. The digest was analysed on an agarose gel.

2.5.6. Ligation of DNA into a plasmid vector

DNA fragments were ligated with T4 DNA ligase in a total volume of 20 μl at 16°C for 16 h using the buffer system supplied by the manufacturer (New England Biolabs). The molar ratio

of vector : insert was 1:1 and 1:5. The concentration of DNA fragments was estimated from the band intensities on analytical agarose gels.

After the ligation the DNA was precipitated and resuspended in a volume of 5 μl dH_2O before electrotransformation. The procedure was as follows: the ligation (20 μl) was mixed with 80 μl dH_2O, 10 μl 3M sodium acetate, pH 5.2 and 88 μl (0.7 - 1.0 volumes) isopropanol. The samples were centrifuged for 30 min in an Eppendorf centrifuge at 14000 rpm and 4°C. The supernatant was discarded and the pellet washed with 70 % ethanol, centrifuged again for 5 min as before and air-dried. The supernatant after centrifugation was discarded. The dry pellet was resuspended in 5 μl dH_2O.

2.5.7. Preparation of electrocompetent *E. coli* cells

1 l LB-medium was inoculated with 10 ml of an *E. coli* (usually strain XL1-Blue) overnight culture and grown to an OD_{600} of 0.6 at 37°C under vigorous shaking. All flasks and solutions subsequently used were sterilized and cooled to 4°C. Cells were centrifuged at 4000 rpm for 15 min and resuspended in 1 l of dH_2O. After a second centrifugation, the cells were resuspended in 500 ml of dH_2O, and centrifuged again. The pellet was then resuspended in 20 ml of 10 % glycerol and centrifugation was carried out. The cells were resuspended to a final volume of 2 - 3 ml in 10 % glycerol. This suspension was distributed in 45 μl aliquots into sterile Eppendorf cups, immediately frozen in liquid nitrogen and stored at - 70°C.

2.5.8. Electrotransformation of *E. coli* cells

For transformation, electrocompetent *E. coli* cells (XL1-Blue) were thawed on ice. 45 μl of cell suspension were mixed with 5 μl of DNA and placed in a pre-chilled sterile electroporation cuvette (EQUIBIO, distance between the electrodes: 2 mm). After a pulse of 2.5 kV, 25 μF and 200 Ω, 950 μl of SOC-medium was added immediately. The cells were gently shaken for 45 min at 37°C and plated on LB-agar plates containing the appropriate antibiotic.

2.5.9. Preparation of SEM-competent *E. coli* cells

For chemical transformations, *E. coli* cells (usually the strains DH5α or BL 21) were taken from a glycerol culture and plated on a LB-agar plate (Inoue et al., 1990). The next day a pre-culture of 3 ml was grown overnight at 37°C under shaking and subsequently, 250 ml of SOB-medium was inoculated. The bacteria were grown to an OD_{600} of 0.6. Then the culture was incubated on ice for 10 min and centrifuged for 10 min, 4°C and 2500 rpm using the

GSA-rotor. The pellet was resuspended in to 4°C pre-chilled TB-solution, incubated on ice for 10 min, centrifuged again and resuspended in 20 ml of TB-solution. 7 % (v/v) DMSO was added and the suspension aliquotted (100 µl) into sterile Eppendorf cups, immediately frozen in liquid nitrogen and stored at - 70°C.

TB-solution: 10 mM PIPES·KOH, pH 6.7, 55 mM $MnCl_2$, 15 mM $CaCl_2$, 250 mM KCl

2.5.10. Transformation of SEM-competent *E. coli* cells

SEM-competent *E. coli* cells were thawed on ice. 100 µl of cell suspension were mixed with 10 ng of plasmid DNA or 5 µl of the ligation. Then, the bacteria were incubated on ice for 30 min, at 42°C for 2 min and put immediately on ice for 5 min. 900 µl of LB-medium was added, and the cups were gently shaken for 45 min at 37°C. The cells were then plated on LB-amp agar plates.

2.5.11. Identification of transformed clones in *E. coli*

Plasmid DNA was prepared from 3 ml overnight cultures (2.5.4.1.) and digested with the appropriate restriction enzymes. Successful transformants were identified by analysing the restriction pattern on an agarose gel.

2.5.12. Filling in DNA 5' overhangs

To fill in 5‘ overhangs to form blunt-ends the DNA polymerase I, large (Klenow) fragment (New England Biolabs, Frankfurt am Main), was used. Blunting was performed strictly following the instructions of the manufacturer. It was essential to take the amount of enzyme indicated per µg of used DNA. The DNA was purified following the protocol of the QIAquick PCR Purification kit or the QIAquick Gel Extraction kit (Qiagen, Hilden).

2.5.13. Phosphorylation and dephosphorylation of DNA

To prevent religation of a cut vector phosphate groups were removed by treatment with calf intestinal phosphatase (CIP). 2.5 µg of linearized vector DNA were incubated in a 25 µl reaction in 1 x CIP buffer (50 mM Tris·HCl, pH 9.0, 1 mM $MgCl_2$, 0.1 mM $ZnCl_2$, 1 mM spermidin) or in NEB buffer 2 - 4 with 1 U CIP for 1 h at 37°C for 5‘ overhangs or for 1 h at 56 °C for blunt-ends and 3‘ overhangs. The reaction was terminated by heating to 68 °C for 5 min in 1/5 volume of 6 x TAE-loading buffer supplemented with 1 % SDS(10 mM Tris·HCl, pH 8.0, 50 mM EDTA pH 8.0, 1 % SDS, 0.1 % (w/v) bromophenol blue, 30 % (v/v)

glycerol). The DNA was purified on an agarose gel or using the QIAquick PCR Purification Kit protocol (Qiagen, Hilden).

For phosphorylation of cut and purified PCR product the DNA was incubated for 30 min at 37 °C with 1/9 volume of 10 x T4 DNA ligase buffer and T4 polynucleotide kinase. After heat inactivation of the enzyme at 65 °C for 20 min the DNA was purified using a sephacryl column (Sephacryl S 300, Pharmacia).

2.5.14. Polymerase chain reaction (PCR)

For preparative and very long PCRs the Expand High Fidelity PCR System (Roche Diagnostics, Mannheim) was used. Generally 25 amplification cycles (denaturing at 94°C for 15 sec, annealing at 58°C for 30 sec, elongation at 72°C for 2 min) were performed after denaturing the reaction at 94°C for 3 min. Elongation time and temperature were adapted to the size of the PCR product according to the instructions of the manufacturer. 50 μl reactions contained 250 μM dNTPs, 25 pmol 5‘ and 3‘ primer, 0.5 U/50μl expand polymerase and 5 μl 10 x PCR reaction buffer. $MgCl_2$-concentration was adapted to improve the quality of the PCR products. The annealing temperature of the primers was generally calculated as 4 x (number of G/C residues) + 2 x (number of A/T residues) - 3.

The PCR products were purified using the QIAquick PCR Purification kit or the QIAquick Gel Extraction kit (Qiagen, Hilden) according to the manufacturer’s manual.

For the analytical amplification of DNA fragments 25 μl reactions containing 20 mM dNTP mix (5 mM for each nucleotide), 25 pmol 5‘ and 3‘ primer, 1 U Taq polymerase and 2.5 μl 10 x PCR buffer (100 mM Tris·HCl, pH 8.3, 500 mM KCl, 15 mM $MgCl_2$, 0.1 % (w/v) gelatine) were taken. λ-phages, cDNA or plasmid DNA were used as templates. In case of λ-phages the reaction was denatured at 94°C for 5 min prior to amplification.

2.5.15. Oligonucleotides

2.5.15.1. Vector primers

Restriction sites are underlined. Stop codons are marked with double lines.

NcKif+2	5‘-P-TGG GGG CAA ATG TTC GGG TG-3‘
NcKif-3	5‘-CGG GAT CCT TAA ATC TGG GCG TCG CGC TCA-3‘
KIFSMe	5‘-TGC CGA ACT CCC TCG ACG TCC-3‘
NcKflHC	5‘-CCA TCG ATA AGC TTT CAC ATG ATA TTA GTC CTC ACC-3‘

NcK433HC 5‘-CCA TCG ATA AGC TTT TAA ATC TGG GCG TCG CGC TCA-3‘

NcKfl_cys 5‘-CCA TCG ATA AGC TTT CAA AAG CAT TTA CGA TGC ACA ATA GAC GGC ATG ATA TTA GTC CTC ACC-3‘

NcK433_cys 5‘-CCA TCG ATA AGC TTT TAA AAG CAT TTA CGA TGC ACA ATA GAC GGA ATC TGG GCG TCG CGC TCA-3‘

2.5.15.2. Sequencing primers

For sequencing of the plasmids the following primers were used:

KIF 200- 5'-GGA TGT TCG TTC TGT TGC CG-3'

KIF 1865+ 5'- CAT GTT TAG ACG CAA GAT CGG-3'

PKL11 5‘-CTC ACT ATA GGG AGA CCA CAA CG-3‘

PK11H 5‘-CGC TGA GAT AGG TGC CTC ACT G-3‘

RO201 5‘-CAT GAT GGG AAC GCC CGA TC-3‘

N7M11 5‘-GTG AAG ATC TGT TCC AGC GCA TTG-3‘

FLMH 5‘-TTC TCC TCC ATC AGC TGC TGC-3‘

S6A7 5‘-CGG TTT CCT GAC GCC CAA CTC-3‘

NcK1 5‘-AAT CTG GGC GTC GCG CTC A-3‘

PO6A1 5‘-GGC AAC CAA GTT GTT CGG-3‘

RKO1 5‘-ATC AAG TCG ATG GCG TCA GC-3‘

T7 5‘-TAA TAC GAC TCA CTA TAG GG-3‘

T3 5‘-AAT TAA CCC TCA CTA AAG GG-3‘ .

Sequencing was performed by the sequencing service of the TOPLAB GmbH, Martinsried, Germany. Primers for sequencing were ordered by the previous and synthesized by the metabion GmbH (Martinsried) or at the TOPLAB GmbH. T7 and T3 primers were standard oligonucleotides and could be ordered for sequencing.

2.5.15.3. NcKin primers

Detection of NcKin in the cDNA libraries was performed with the following primers:

NKNde5 5’-CGG GAG CCA TAT GTC GTC AAG TGC G-3’

NK433- 5'-AAC TGC AGT TAA TCC AAG GGC AAG CTG GG-3'

2.5.16. Isolation of RNA from *N. crassa*

Mycelia from 100 ml of a minimal medium culture grown under standard condititions (2.4.2.) were harvested and immediately frozen in liquid nitrogen. The frozen mycelia were ground with sand and liquid nitrogen. The powder was given to a solution of phenol and RNA extraction buffer and mixed immediately. The samples were shaken for 10 min at room temperature, centrifuged for 30 min, 4°C, in an Eppendorf centrifuge at maximum speed and the supernatant was removed to chloroform/isoamylalcohol (Ready Red, Appligene), mixed, shaken for 5 min and centrifuged for 20 min, 4°C, as before. The supernatant was combined with 1/10 volume of 3 M sodium acetate, and ice cold 100 % ethanol (RNase free) was added. The samples were incubated at -20°C over night. After a centrifugation of 30 min as described before, the pellet was washed with ice cold 70 % ethanol and dried under vacuum. The pellet was suspended very carefully in an appropriate amount of ice cold dH_2O, and the concentration was determined.

RNA extraction buffer: 1 mM EDTA, pH 8.0, 100 mM Tris·HCl, pH 8.0, 4 % (w/v) SDS, 0.6 M NaCl

2.5.17. Electrophoresis of RNA and Northern blotting

20 μg of total RNA in dH_2O were mixed to an end concentration of 1 x MOPS, 0.8 M glyoxal and 50 % v/v DMSO. The samples were denatured for 30 - 60 min at 50 °C and following put on ice. 1/4 volume of loading buffer (1 x MOPS, 50 % glycerol, 5 ‰ bromophenol blue, 5 ‰ xylene cyanol) was added and the samples loaded on a 1 % 1 x MOPS-agarose gel. Electrophoresis was performed for 2 h with 90 V. Before blotting the gel was equilibrated in 20 x SSC for 10 min and blotted onto a nylon membrane (Hybond NX, Amersham) in 20 x SSC by capillary transfer. The blot was air-dried and the RNA cross-linked by UV-illumination. To visualize RNA bands the membrane was stained for 5 min in 200 mg/l methylene blue in 0.3 M sodium acetate, destained with dH_2O and imaged next to a ruler.

20 x SSC buffer: 3 M NaCl, 0.3 M sodium citrate x 2 H_2O; pH to 7.0 with HCl

10 x MOPS buffer: 200 mM MOPS, 80 mM sodium acetate x 3 H_2O, 10 mM Na_2-EDTA x 2 H_2O; pH to 7.0 with 5 M NaOH

2.5.17.1. Construction of the probes for Northern blotting

For the detection of NcKIF1 a PCR from the λ10 vector (Seiler S., 1999) using the primers NcKif+2 and NcKif-3 was performed and purified by gel extraction. This PCR has a size of 1208 bp containing the conserved motor domain and the following 14 amino acids.
As probe for NcKin pT12-1 was cut with *Hind* III. The fragment containing the tail of NcKin was excised from an agarose gel and purified as described before.

2.5.17.2. Radioactive labelling of the probes, hybridization and detection

The DNA probes for hybridization were synthesized with the Top-Label-Kit (New England Biolabs) according to the instructions of the manufacturer. This method involves hybridisation of random oligonucleotides to a single strand DNA probe and synthesis of the complementary strand by the Klenow fragment, incorporating [α-^{32}P]-dCTP (10 μCi/μl). Nucleotides that had not been incorporated were removed by centrifuging through a Micro Spin™ S300 HR-column (Amersham). About 200 ng of DNA were used.
The probe was denatured for 5 min at 100°C prior to hybridisation and mixed with 20 ml hybridisation buffer. The nylon membrane was pre-hybridised with hybridisation buffer for 1 h at 60°C before addition of the DNA probe. Hybridisation was performed at 60°C over night and the filter washed twice for 20 min at 60°C in wash buffer. The membrane was wrapped in foil and exposed on a phosphoimager for 3 days.

Hybridisation buffer: 50 mM Na-phosphate buffer, pH 7.0, 50 mM PIPES, pH 6.5, 100mM NaCl, 5 % (w/v) SDS, 1 mM EDTA, pH 8.0

Wash buffer: 1 x SSC, 5 % SDS

2.5.18. Screening of cDNA

For the screening of λZAP libraries XL1-Blue MRF‘ cells were grown overnight in LB-medium containing 0.2 % (w/v) maltose and 10 mM $MgSO_4$, harvested and resuspended in about 15 ml of 10 mM $MgSO_4$. The cells were diluted to an OD_{600} of 0.5 with 10 mM $MgSO_4$. 600 μl of the bacterial suspension were infected with 50000 pfu of the phage library and agitated for 15 min at 37°C. The suspension was plated with 6.5 ml of NZY top agar on 150-mm NZY agar plates. The plates were inverted and incubated at 37°C for 8 h. Before placing the filters the plates were chilled for 2 h at 4°C to prevent the NZY top agar from sticking to the membrane. Nylon membranes (Hybond N, Amersham) were cut to the size of the plates and put for 1 min onto the agar plates. The position of the membranes was labelled by

piercing with a needle. The filters were placed with the phage side up on Whatman 3 MM filters soaked with 0.5 M NaOH and 1.5 M NaCl denaturation solution for 5 min. The filters were then neutralized by treating them the same way with 1 M Tris·HCl, pH 8.0, and 1.5 M NaCl neutralization solution and finally for 2 min with 2 x SSC. After air-drying the DNA was UV cross-linked, filters were hybridized with DIG-labelled probes and colour detection was carried out as described in 2.5.17.1.. Positive plaques were excised, vortexed with 500 μl SM-buffer/20 μl chloroform and incubated for 1 - 2 h at room temperature or overnight at 4°C to allow the phages to diffuse into the buffer. Screening was repeated with the excised phages, reducing the number of pfu in each round, until all phages were positive.

The pBluescript plasmids containing the cDNA were rescued by in vivo excision. For this, XL1-Blue MRF‘ and SOLR cells were grown in LB-medium, supplemented with 0.2 % (w/v) maltose and 10 mM $MgSO_4$, gently spun down and resuspended at an OD_{600} of 1.0 in 10 mM $MgSO_4$. 200 μl of these XL1-Blue MRF‘ cells were mixed with 250 μl of phage stock and 1 μl of ExAssist helper phage (Stratagene) and incubated at 37°C for 15 min. 3 ml of LB-medium were added and the solution incubated for 2.5 - 3 h at 37°C with shaking. The samples were heated at 65 - 70°C for 20 min and centrifuged at 1000 x g for 15 min. 100 μl and 10 μl of the supernatant were mixed with 200 μl freshly grown SOLR cells (as described above), incubated at 37°C for 15 min and plated on LB-amp agar plates. Colonies of SOLR cells containing the excised pBluescript-phagemid were picked and analysed.

NZY-medium:	5 g/l NaCl, 2 g/l $MgSO_4$ x 7 H_2O, 5 g/l yeast extract, 10 g/l NZ amine (casein hydrolysate), pH 7.5
NZY-agar:	15 g/l agar in NZY-medium
NZY top agar:	0.7 % (v/w) agarose in NZY-medium
SM-buffer:	5.8 g/l NaCl, 2 g/l $MgSO_4$ x 7 H_2O, 50 mM Tris·HCl, pH 7.5, 0.01 % (w/v) gelatin

2.5.18.1. Digoxygenin labelling of the probes, hybridisation and detection

For the detection of the NcKIF1-sequence the DNA probe was labelled with DIG using the DIG DNA labelling and detection system (Boehringer Mannheim). As probe the same PCR-probe as described for NcKIF1 in section 2.5.17.1. was used. The DIG-labelled DNA probe was synthesized by PCR using a 20 x dNTP mix (see section 2.5.13.) supplemented with an equal volume of 10 x DIG DNA labelling mix (same total dNTP concentration). The labelled

probe, which runs slower than the corresponding unlabelled PCR fragment in the gel electrophoresis, was purified by gel extraction, and concentration was determined.

The nylon membranes with the cross-linked DNA were pre-hybridised in Easy Hyb solution (Boehringer Mannheim) at 37 °C for 1 h and were then supplemented with the freshly denatured DIG-labelled probe (20 ng/ml or 0.8 μg/40 ml Easy Hyb solution). The DIG-labelled probe was denatured at 100°C for 5 min and put on ice immediately. Hybridisation was performed at 37°C over night and the membranes washed twice with high salt buffer (2 x SSC, 0.1 % SDS) for 5 min at room temperature and twice with low salt buffer (0.1 x SSC, 0.1 % SDS) for 15 min at 65°C.

The membranes were equilibrated with maleic acid buffer (100 mM maleic acid, 150 mM NaCl, pH 7.5) for 1 min, blocked for 30 min with blocking buffer (maleic acid buffer containing 1 % blocking reagent) and incubated with anti-digoxigenin antibodies coupled to alkaline phosphatase (1:5000 dilution in maleic acid buffer containing 1 % blocking reagent) for 30 min at room temperature. Unbound antibodies were removed by two washes for 15 min in maleic acid buffer, and the membranes were equilibrated for 2 min with the following solution: 100 mM Tris·HCl, 100 mM NaCl, 50 mM $MgCl_2$, pH 9.5. Then the probe was detected by NBT/BCIP colour detection. For this, NBT/BCIP-Ready-to-use-tablets (Roche) were dissolved in dH_2O (1 tablet/10 ml dH_2O). The membranes were incubated at room temperature for 10 min – 1 h in the dark without any movement. To stop the reaction the membranes were washed in dH_2O and dried subsequently.

2.5.19. Construction of the vectors

NcKIF1 399

A PCR from the λ10 vector (Seiler S., 1999) using the primers NcKif+2 and NcKif-3 was performed. pT7-7 was cut with *Nde* I and the 5'-overhang filled-in with Klenow polymerase. The vector as well as the PCR were cut with *Bam*H I and ligated. The first base pair (A from ATG) was delivered by the pT7-7, the next two by the oligonucleotide NcKif+2. The start codon was introduced artificially, because the sequence was not complete.

NcKIF1 434, NcKIF1 434cys, NcKIF1 647 and NcKIF1 647cys

After screening of a mycelial cDNA library (FGSC) a PCR from the positive and sequenced cDNA clone 7A.1 was performed. The following primers were used: KIFSMe/NcK433HC for NcKIF1 434, KIFSMe/NcK433_cys for NcKIF1 434cys, KIFSMe/NcKflHC for NcKIF1 647 and KIFSMe/NcKfl_cys for NcKIF1 647cys. With the primers NcK433_cys and NcKfl_cys a

cys-tag, amino acids PSIVHRKCF, (Itakura et al., 1993) was introduced C-terminally for protein modifications.

pT7-7 was cut with *Nde* I and the 5'-overhang filled-in with Klenow polymerase. The vector and the PCRs were cut with *Cla* I or *Bsp*D I and ligated. *Bsp*D I is an isoschizomer of *Cla* I. The first base pair from the start codon (ATG) is delivered by the pT7-7 vector. The primer KIFSMe starts with the second base pair from the NcKIF1-sequence (TGCCGAA...).

2.6. Biochemical methods

2.6.1. SDS-Polyacrylamide gel electrophoresis (PAGE)

Proteins were separated by discontinuous SDS-Page (Laemmli, 1970). For this purpose 10 % polyacrylamide gels were prepared. The gels were run with a minigel-system (Hoefer Scientific Instruments, San Francisco, USA). Protein samples were mixed with 1/5 volume of 6 x Laemmli sample buffer and heated at 95°C for 5 – 10 min prior to use. As molecular weight standard, the „High molecular weight" (Sigma) was used. Samples and standard were stored at –20°C.

PAA-solution:	30 % acrylamide, 0.8 % bisacrylamide
Running buffer:	25 mM Tris·HCl, 0.1 % SDS, 192 mM glycine
Upper stock:	500 mM Tris·HCl, pH 6.8, 0.4 % SDS
Lower stock:	1.5 M Tris·HCl, pH 8.8, 0.4 % SDS
6 x Laemmli sample buffer:	300 mM Tris·HCl, pH 6.8, 15 mM EDTA, 12 % SDS, 30 % glycerol, 15 % β-mercaptoethanol, 0.06 % bromophenol blue

2.6.2. Coomassie staining

Gels were stained for at least 4 h in Coomassie staining solution and after that destained in Coomassie destaining solution. The gels were scanned for documentation and dried between two sheets of cellophane wrap stretched by a plexiglas frame.

Coomassie staining solution:	50 % methanol, 10 % acetic acid, 0.25 % Coomassie Brilliant Blue R 250 (Sigma)

Coomassie destaining solution: 10 % acetic acid in dH_2O

2.6.3. Determination of protein concentration

For the determination of protein concentration the Bradford assay (Bradford, 1976; BioRad Inc.) was used. For each measurement, a reference curve with BSA was generated. The data were evaluated with the KaleidaGraph programme (Macintosh).

2.6.4. Purification of tubulin

Tubulin was purified using three successive steps of polymerisation and depolymerisation followed by an ion exchange chromatography step (according to Mandelkow et al., 1985). Pig brain halves were obtained at the local slaughterhouse and put on ice immediately. They were separated from blood vessels and meninges as quickly as possible. To 700 g of brain, 700 ml of buffer A was added. The mixture was homogenized in a blender (Braun) and centrifuged for 90 min at 4°C with 12000 rpm using the GSA rotor. To the supernatant 25 % glycerol (v/v) and 2 mM ATP (each final concentration) were added. The tubulin was polymerised at 35°C for 30 min in a waterbath under gentle agitation. The microtubules were sedimented at 32°C (prewarmed centrifuges and rotors, Beckman rotor 35, 35000 rpm, 60 min and Beckman rotor TI 45, 42000 rpm, 45 min). The pellets were resuspended on ice in 100 ml of buffer C using two 50 ml glas homogenizers (Wheaton). Afterwards, the microtubules were depolymerised for 25 min on ice and centrifuged again (precooled centrifuge and Beckman rotor 42.1, 4°C, 36000 rpm, 30 min). The supernatant was polymerised at 35°C for 30 min in a waterbath under addition of ATP to a final concentration of 2 mM and pelleted again (prewarmed centrifuge and Beckman rotor 42.1, 32°C, 33000 rpm, 60 min). The pelleted microtubules were pooled, weighed, frozen in liquid nitrogen and stored at –70°C until further purification.

Phosphocellulose (P11, Whatman) was activated as indicated. 50 ml of P11 material was packed into a column (1.5 x 10 cm). For equilibration, the column was connected to the FPLC and 3 column volumes of buffer D at a flow rate of 1 ml/min were run through. At the same time, 10 – 20 g of microtubules were thawed, homogenized in 50 – 100 ml of buffer B in a glas homogenizer and depolymerised for 25 min on ice. After centrifugation (precooled centrifuge and Beckman rotor 42.1, 4°C, 36000 rpm, 30 min) the supernatant was supplemented with 10 % DMSO and 2 mM ATP (each final concentration), and the tubulin polymerised at 35°C for 30 min. The microtubules were pelleted (prewarmed centrifuge and Beckman rotor 42.1, 32°C, 33000 rpm, 60 min), resuspended in 5 – 7 ml of buffer D, again homogenized and depolymerised on ice for 30 min. The tubulin solution was centrifuged (precooled centrifuge and Beckman rotor TI 70.1, 34200 rpm, 4°C, 30 min) and the

supernatant loaded onto the phosphocellulose column. After the sample had entered the column completely, buffer D was loaded to the column and the protein eluted at a flow rate of 0.2 ml/min. 1.5 ml-fractions were collected in a fraction collector. The peak fractions were identified using the Bradford reaction (see 2.6.3.). For this, 2 μl of the fraction were added to 500 μl of the Bradford mixture (100 μl of Bradford reagent, 400 μl of dH_2O). The darkest fractions were pooled and frozen in liquid nitrogen after addition of 0.1 mM GTP. The tubulin was stored at –70°C.

Buffer A: 100 mM PIPES·NaOH, pH 6.9, 2 mM EGTA, 1 mM $MgSO_4$, 0.1 mM ATP, 1 mM DTT

Buffer B: 500 mM PIPES·NaOH, pH 6.9, 1 mM EGTA, 1 mM $MgSO_4$, 1 mM ATP, 1 mM DTT

Buffer C: 100 mM PIPES·NaOH, pH 6.9, 1 mM EGTA, 1 mM $MgSO_4$, 1 mM ATP, 1 mM DTT

Buffer D: 100 mM PIPES·NaOH, pH 6.9, 1 mM EGTA, 1 mM $MgSO_4$, 0.05 mM ATP, 1 mM DTT

ATP and DTT were added to the buffers on the day of use and the pH determined.

2.6.5. Polymerisation of microtubules

Tubulin (see 2.6.4.) was thawed and centrifuged (rotor TLA 100.3, 4°C, 80000 rpm, 10 min). GTP was added to a final concentration of 1 mM and the tubulin polymerised for 30 min at 37°C. After 5 - 10 min of polymerisation time, 20 μM taxol (final concentration) was added for stabilization of the microtubules. Centrifugation was carried out through 40 % sucrose (w/v) in ATPase buffer supplemented with 20 μM taxol, final concentration, (see 2.6.11.2.) for 10 min at 25°C, rotor TLA 100.3 and 80000 rpm. The pellet was washed with ATPase buffer/20 μM taxol and resuspended in 1/5 volume of this buffer. After that, the microtubule concentration was determined, if required.

2.6.6. Determination of microtubule concentration

The microtubule concentration was determined after denaturation in guanidine·HCl. The microtubules were diluted 1:5 and 1:10 in the used buffer supplemented with taxol. 10 μl of this dilution was added to 90 μl of 6.6 M guanidine·HCl, and the OD_{280} was measured. As reference value 10 μl of the used buffer/taxol were added to 90 μl of guanidine·HCl. The microtubule concentration was calculated as follows (Huang and Hackney, 1994):

Microtubules (μM) = (A_{280}/1.03) · 10 · dilution of the microtubules

The mean value of the two dilutions was taken.

2.6.7. Expression and purification of recombinant NcKIF1 from *E. coli*

For the expression of recombinant proteins TPM-medium supplemented with 10 mM glucose and 100 μg/ml ampicillin for the *E. coli* strain BL 21 or 100 μg/ml ampicillin and 25 μg/ml chloramphenicol for the *E. coli* strain BL 21 RIL was taken. TPM-medium was inoculated directly with a colony from an agar plate or 20 ml of a preculture, which was grown for 16 h at 37°C and 240 rpm. The bacteria were grown at 22°C and 240 rpm to an OD_{600} of 0.6 and then induced with 500 μM IPTG. After 2 h – 23 h induction time the bacteria were harvested (Beckman J6-HC centrifuge, 4°C, 35 min). 2–5 g of bacteria were resuspended in AP-100 supplemented with protease inhibitors (1 x Pi in AP-100; 200 μM Pefabloc (Pe), Roche Diagnostics) and 1 mM DTT. After the bacteria have dissolved completely DNAse I was added to the solution. The bacteria were lysed by sonication, centrifuged at 4°C (Ti 42.1, 30 min, 42000 rpm) and the supernatant diluted four times to 25 mM PIPES·KOH final concentration with dilution buffer. 1 mM DTT and 10 μM ATP was added to the buffer before. For purification an S-sepharose column (Amersham HiTrap™ SP HP) was connected to the FPLC and equilibrated with buffer A, supplemented with 1 mM DTT and 10 μM ATP. Elution took place by stepwise addition of buffer B. This buffer was also supplemented with 1 mM DTT and 10 μM ATP. The recombinant NcKIF1 was eluted at 300 mM NaCl concentration. The peak fractions were pooled, 10 % of glycerol added, divided into aliquots, frozen in liquid nitrogen and stored at –70°C. For protein preparations containing no DTT, this agent was just added to the AP-100 buffer.

TPM-medium: 20 g/l tryptone, 15 g/l yeast extract, 8 g/l NaCl, 2.5 g/l Na_2HPO_4 x 2 H_2O, 1 g/l KH_2PO_4

AP-100: 100 mM PIPES·KOH, pH 6.9, 2 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA; pH 6.9

100 x Pi: 1 mg/ml soybean-trypsin-inhibitor, 1 mg/ml TAME, 250 μg/ml leupeptin, 100 μg/ml pepstatin A, 100 μg/ml aprotinin

Buffer A: 25 mM PIPES·KOH, pH 6.9, 1 mM EGTA, 1 mM $MgCl_2$; pH 6.9

Buffer B: 25 mM PIPES·KOH, pH 6.9, 1 mM EGTA, 1 mM $MgCl_2$, 1 M NaCl; pH 6.9

Dilution buffer: 1 mM EGTA, 1 mM $MgCl_2$; pH 6.9

2.6.7.1. Expression studies

To test, if the kinesin is expressed, NcKIF1 399 was expressed in BL 21 with 500 μM IPTG for 3 h and 23 h at 37°C and 22°C. 1 ml was taken from the cultures and the OD_{600} measured. To get comparable results for protein expression, the volumes of the samples were adjusted to the OD_{600} (concentration of the bacteria), corresponding 1 ml of bacterial suspension to an OD_{600} = 0.1. The calculated volumes were centrifuged in an Eppendorf centrifuge for 1 min at 14000 rpm, the supernatants discarded and the pellets resupended in 1 x Laemmli sample buffer. The samples were heated at 95°C for 5 min. SDS-PAGE and Western blot were performed.

For testing the solubility of the expressed protein, NcKIF1 399 was expressed in BL 21 with 500 μM IPTG for 23 h at 22°C and 37°C. The bacteria were harvested and 0.5 g resuspended in 2.5 ml of AP-100 supplemented with 1 mM DTT and protease inhibitors as described above (see 2.6.7.). A sample was taken, 1/5 volume of 6 x Laemmli sample buffer added and heated at 95°C for 5 min. The bacteria were lysed by sonication and centrifugation was carried out (TLA 100.3, 4°C, 80000 rpm, 20 min). A sample was taken before centrifugation and from the supernatant after centrifugation. Treatment to the samples was the same as described before. The samples were examined on a PAA-gel and Western blot performed.

In a further experiment, induction was tested with 100 μM and 500 μM IPTG at 22°C for 24 h. Before and after induction 1 ml of the culture was taken and the OD_{600} measured. As a control, a culture of BL 21 containing no plasmid was grown also but not induced. The 500 μM IPTG-induced culture was lysed and centrifuged as described in 2.6.7.. A sample of the supernatant after centrifugation was taken, 1/5 volume of 6 x Laemmli sample buffer added and heated at 95°C for 5 min. SDS-PAGE was performed.

The expression of NcKIF1 647 yielded a degraded protein. To try to improve the expression pattern different *E. coli*-strains (BL 21, BL 21 RIL, MC1061) were tested. For the cultivation of MC1061 kanamycin and ampicillin were used as antibiotic supplements. Expression was performed at 22°C and 500 μM IPTG. Certain times after induction (2 , 4 , 6 , 14 or 16 h) the bacteria were harvested. Before induction and after the induction time a sample was taken. The OD_{600} was measured and the volume of the bacterial suspension calculated as decribed above. The calculated volumes were centrifuged in an Eppendorf centrifuge for 1 min at 14000 rpm, the supernatants discarded and the pellets resupended in 1 x Laemmli sample buffer. The samples were heated at 95°C for 5 min. Furthermore, the S2 of the harvested

bacteria (supernatant after sonication and ultracentrifugation) was prepared (see 2.6.7.), 1/5 volume of 6 x Laemmli sample buffer added and heated at 95°C for 5 min. The samples were examined on a PAA-gel and Western blot performed.

2.6.7.2. Testing of the purification conditions of recombinant NcKIF1

To test the purification conditions, 2g of BL 21 after expression of NcKIF1 399 was dissolved in 10 ml of AP-100 + Pe, Pi and DTT (see 2.6.7.) on ice. The following procedure was the same as described in section 2.6.7.. 3 ml of SP-sepharose and 3 ml of DEAE-sephadex were loaded into emptied Qiagen Midi-Prep. columns and equilibrated with buffer A + 1 mM DTT and 10 μM ATP. The diluted supernatant was split into two parts and loaded onto the two columns. The flow through was collected. Then, elution took place with 50, 300 and 1000 mM NaCl-elution buffer (see 2.6.7.). 5 1 ml-fractions were collected. The eluted fractions as well as the flow through were tested for activity in the ATPase assay (see 2.6.13.2.).

2.6.8. Density gradient centrifugation and gel filtration

For determination of the oligomerization state of NcKIF1 density gradient centrifugation and gel filtration were performed (Cantor and Schimmel, 1980). The first was performed using 5 - 18 % (w/v) sucrose density gradients. 5, 7, 9, 11, 13 and 18 % sucrose gradients were mixed in 300 mM NaCl-elution buffer (see 2.6.7.) on ice. 10 μM ATP was added to the mixture, and 600 μl of each were put in a centrifuge tube starting with the highest gradient. The tubes were stored at 4°C for at least 4 h in upright position to get an equal and reproducible gradient. Then, a mix of 400 μl containing the previous buffer, aldolase (0.5 mg/ml; $S_{w,20}$=7.4), carboanhydrase (0.5 mg/ml; $S_{w,20}$=3.2), BSA (0.3 mg/ml; $S_{w,20}$=4.22), kinesin (0.5 mg/ml) and 10 μM ATP was bedded over the gradient. The sample was centrifuged overnight (13 - 16 h, 4°C, 37000 rpm, rotor Sw50.1.). Fractions of 300 μl were collected from the bottom using a fraction collector, SDS-PAGE (2.6.1.) performed and the gel analysed using the NIH Image programme (Macintosh). From the position of the reference proteins a standard curve was drawn (KaleidaGraph, Macintosh) and the $S_{w,20}$-value of the kinesin determined.

For gel filtration, a Superdex 200 gel filtration column (Pharmacia Biotech) was connected to the FPLC and equilibrated with 300 mM NaCl-elution buffer containing 10 μM ATP at a flow rate of 0.2 ml/min. A 500 μl-mix of standard proteins, ferritin (450 kDa; r_{Stokes}=5.9 nm), aldolase (158 kDa; r_{Stokes}=4.5nm), BSA (68 kDa; r_{Stokes}=3.55 nm), carboanhydrase (29 kDa; r_{Stokes}=2.4 nm) and cytochrome C (12.5 kDa; r_{Stokes}=1.64 nm), in 300 mM NaCl-elution buffer was loaded onto the column and eluted at a flow rate of 0.3 ml/min. Then, a sample of 500 μl of the protein (A_{280} approx. 0.5, diluted in elution buffer containing 10 μM ATP, if necessary)

was loaded and eluted at the same flow rate. The elution of the proteins was monitored by an UV-cell and a chart recorder (0.5 cm/ml). The elution volume of the standard proteins was compared to that of the examined protein and the Stokes radius determined from a regression line (Andrews, 1970). The elution fractions were collected and analysed in an SDS-PAGE.

The native molecular weight of a protein can be calculated from its Stokes radius and its $S_{w,20}$-value as follows:

$$M_r = (S_{w,20} \cdot n_A \cdot 6\pi \cdot \eta \cdot r_{Stokes}) / (1-\upsilon \cdot \rho)$$

with

$S_{w,20}$	sedimentation coefficient in H_2O at 293 K	$[10^{-13}$ s]
M_r	molecular weight	$[g \cdot mol^{-1}]$
n_A	Avogadro's number	6.023×10^{23} mol^{-1}
υ	specific volume of the protein	0.725 $cm^3 \cdot g^{-1}$
ρ	density of H_2O at 293 K	1 $g \cdot cm^{-3}$
η	viscosity of H_2O at 293 K	10^{-3} $N \cdot s \cdot m^{-2}$
r_{Stokes}	Stokes radius	[nm]

A rearrangement yields:

$$M_r = 4128 \times S_{w,20} \times r_{Stokes} \; [g \cdot mol^{-1}]$$

2.6.9. NEM-inhibition test

To test, if the kinesin contains a reactive cysteine besides that introduced by the cys-tag, which could also be tagged unintentionally, the NEM-inhibition test was performed. For the test NcKIF1 434 was selected, because this construct does not attach to the glass surface in the multiple motor gliding assay, and therefore had to be biotinylated to attach the construct using streptavidin (see 2.6.11. and 2.6.12.).

To remove the DTT from the NcKIF1 434 solution gel filtration using MicroSpin™ G-25 columns (Amersham) was performed. The procedure was performed at 4°C. The column was shortly centrifuged. Unspecific binding sites were blocked by loading 500 μl (a single column volume) of kinesin dilution buffer (see 2.6.13.2.) onto the column and let the buffer infiltrate the resin for 5 min. Then, the buffer was washed out with 5 column volumes of 300 mM NaCl-elution buffer (see 2.6.7.) supplemented with 100 μM ATP by loading the column 5 times and centrifuging at 3000 rpm in an Eppendorf centrifuge. 50 μl of the kinesin solution

was loaded onto the column and centrifuged for 2 min as above. The protein concentration of the flow through containing the purified kinesin as well as the kinesin solution before gel filtration were determined using the Bradford assay (see 2.6.3.). The activity of the eluate was measured in the ATPase assay (see 2.6.13.2.).

For the test, 100 μM NEM was added to 2.34 μM NcKIF1 434 (about 40-fold excess) and incubated on ice for 5 min, 15 min, 30 min and 1 h. After these times, the activity of the protein was measured in the ATPase assay. As a control, 1 mM NEM was inactivated with 10 mM DTT and added to the kinesin as above. The activity of the protein was measured after 30 min and 60 min. The data were evaluated using the KaleidaGraph programme (Macintosh). The NEM was inactivated by the addition of a 10-fold molar excess of DTT.

2.6.10. Cy3-labelling of cys-tagged NcKIF1 constructs

As a further test to determine how many reactive cysteines a protein contains Cy3-labelling can be performed, and the number of bound Cy3 can be determined in relation to the kinesin molecules.

First, Cy3-succimide was converted into Cy3-maleimide. For this, 2.0 mg of PEM (Dojindo Laboratories) was dissolved in 2 ml of DMSO. 24.5 μl of this solution (corresponds to 100 μMol of PEM) was added to a tube of Cy^{TM}3 monofunctional reactive dye (Amersham). The tube was wrapped into aluminium foil and incubated at 40°C for 12 h (Pierce et al., 1998). Microtubules were polymerised as described in section 2.6.5., but instead of ATPase buffer BRB80 was used. The concentration of the microtubules was determined as described in 2.6.6.. For the determination of the Cy3-concentration the Cy3-solution was diluted 1:1000 in BRB80 and the A_{552} measured. For the Cy3-labelling NcKIF1 434cys (a DTT-free preparation) was taken. Cy3-maleimide was added to the kinesin solution at a 6-fold molar excess and incubated for 1 h on ice. The reaction was stopped by addition of 10 mM DTT. The following procedure was the same as described in section 2.6.11.. Incubations took place in the dark to avoid bleaching of the dye. The release step was repeated with release buffer containing 400 mM KCl. The releases were measured in the photometer at A_{552} for the Cy3-concentration. The activity was measured with the ATPase assay (see 2.6.13.2.). The protein concentrations were determined with the Bradford assay [mg/ml] (see 2.6.3.). The releases were frozen in small aliquots under addition of 10 % glycerol in liquid nitrogen, wrapped into aluminium foil and stored at -70°C.

For the calculation of how many Cy3-molecules have bound to the kinesin the first release was used. The calculation was performed as follows: The A_{552} for the Cy3 concentration was

1.537. With the Bradford assay a protein concentration of 0.58 +/- 0.18 mg/ml was measured. First, the molar concentration of NcKIF1 434cys in the release was calculated. The by the GCG programme PEPTIDESORT (Wisconsin Package Version 10.2, Genetics Computer Group (GCG), Madison, Wisc.) calculated number of 49.546 kDa for the molecular weight of NcKIF1 434cys was used for the calculation. The protein concentration [g/l] was divided by the molecular weight [Da; 1 Da = 1 g/mol] resulting in a molar concentration of 11.7 μM for NcKIF1 434cys in the release. The molar concentration of Cy3 in the release [μM] was calculated after the Lambert-Beer law, $A = \varepsilon \cdot c \cdot d$, (see 2.6.13.3.) with the value for Cy3 at A_{552}, d (thickness of the cuvette) = 1 cm and ε_{552} for Cy3 = $130 \cdot 10^3$ $M^{-1} \cdot cm^{-1}$. The concentration of Cy3 in the release was 11.8 μM. Setting the molar concentration of Cy3 (11.8 μM) in relation to that of the protein (11.7 μM), it can be concluded that one Cy3-molecule has bound to one kinesin molecule. The calculations were also performed with the values for the second release and support this result.

2.6.11. Biotinylation of cys-tagged NcKIF1 constructs

The biotinylation was performed for the multiple motor gliding assay (see 2.6.10.) using the streptavidin-biotin system. For this, the reactive cysteine residue in the cys-tag can be conjugated with biotin using biotin-maleimide (Funatsu et al., 1997, Inoue et al., 1997, Itakura et al., 1993).

Biotin-maleimide („Long chain“ biotin-maleimide, B-1267, Sigma) was solved in DMSO to a final concentration of 10 mM and added to the kinesin solution at a 6-fold molar excess. The final concentration of DMSO should be under 1 % in the solution. The mix was incubated for 1 h on ice, the reaction stopped by addition of 10 mM DTT (final concentration) and aggregates removed by centrifugation (TLA 120.1, 4°C, 100000 rpm, 10 min). Microtubules were polymerised (see 2.6.5.). Instead of ATPase buffer BRB80 (see 2.6.10.) supplemented with 20 μM taxol was used for this. The active kinesin was isolated by a microtubule binding and release step (Vale et al., 1985b). Microtubules were added to the kinesin solution at a 3 – 4-fold molar excess and incubated under addition of 1 mM AMP-PNP for 15 min at room temperature. Unbound biotin was removed by centrifugation through a sucrose cushion (40 % sucrose in BRB80; TLA 120.1, 25°C, 80000 rpm, 10 min), and the pellet containing the bound kinesin resuspended in 100 μl of release buffer (BRB80, 20 μM taxol, 300 mM KCl, 5 mM ATP) to release the kinesin. The sample was incubated for 10 min at 22°C, and the microtubules pelleted by centrifugation (TLA 120.1, 25°C, 80000 rpm, 10 min). The supernatant containing the biotin-kinesin conjugate was taken, put on ice, 10 % glycerol

added, aliquotted into 10 μl, frozen in liquid nitrogen and stored at –70°C. The release was analysed in an SDS-PAGE and the protein concentration determined using the Bradford assay (see 2.6.3.).

2.6.12. Multiple motor gliding assay

To test the motility of the NcKIF1 constructs a multiple motor gliding assay was performed. For this, a flow chamber was used (Vale et al., 1985a). This flow chamber consists of a slide on which a coverslip was attached by two stripes of double faced tape. The space between them can be floated by different solutions. To make the motility assay possible for the short construct (NcKIF1 434) the biotinylated kinesin (see 2.6.9.) was attached to the glass surface by streptavidin. This was also performed with NcKIF1 647 to see, if the protein solution may be purified from the degradation products. If so, just the complete proteins would contribute to the observed movements, and not complete ones would be washed away.

Microtubules were polymerised as described in section 2.6.5. using BRB80/taxol in the procedure. The flow chamber was incubated for 10 min with 1 mg/ml streptavidin in BRB80, washed with the three fold amount of BRB80 containing 1 mg/ml BSA and after that incubated for 10 min with the kinesin in BRB80, 1 mg/ml BSA and 0.8 mg/ml casein. The flow chamber was washed with the three fold amount of BRB80 under addition of 0.8 mg/ml casein. Then, microtubules, 0.8 mg/ml casein, 10 mM ATP, 10 mM $MgCl_2$ and 100 mM KCl in BRB80 were floated into the chamber. The movements of the microtubules were observed with a Zeiss Axiophot microscope (Zeiss, Oberkochen) in differential interference contrast and a Hamamatsu C2400-7 Camera. The image was transferred via a „DVS-1000 Image processing System“ (Hamamatsu) to a monitor (Sony) in a black and white mode. The movements were taped on videotape (Fuji). To measure velocities, two points were marked on the screen and the time which was needed for a microtubule to travel from one point to the other was recorded. The distance was taken from a scale that had been obtained by gauging the screen with an object micrometer.

For determination of the Michaelis-Menten constant (K_m) for ATP different ATP concentrations were taken keeping the other parameters constant. A phosphoenolpyruvate / pyruvate kinase-regenerating system for ATP was used in this assay. For this, 2 mM phosphoenolpyruvate (mono-potassium salt, Sigma) and 1.5 μl (1:10 in BRB80/100 mM KCl) pyruvate kinase (same units as in the ATPase assay, see 2.6.11.2.; Roche) were added to the assay together with the microtubules. For testing the dependence of the velocity on ionic strength different KCl-concentrations were taken keeping the other parameters constant.

For the conventional multiple motor gliding assay (Paschal and Vallee, 1993) 5 μl of motor solution were put on a coverslip and incubated for 5 min in a wet chamber to let the motor attach to the glass surface. After that, microtubules, 10 mM ATP, 10 mM $MgCl_2$ and 100 mM KCl were added. The coverslip was put onto a glass slide and sealed with 1:1:1 of paraffin, lanolin and vaseline. The microtubules were observed as described above.

BRB80: 80 mM PIPES·KOH, pH 6.9, 5 mM $MgCl_2$, 1 mM EGTA

2.6.13. ATPase assay

2.6.13.1. Basal activity of kinesin

The basal ATPase activity (k_0) in the absence of microtubules was measured using radioactive [γ-^{32}P]-ATP (Shimizu et al., 2000). Radioactive [γ-^{32}P]-ATP (Amersham/Pharmacia) was diluted 1:1000 in 0.1 M ATP, pH 7 (approx. $3.7 \cdot 10^6$ Bq/ml). In the assay 0 μM, 1 μM and 2 μM of kinesin (NcKIF1 434) were used in each reaction. The kinesin was added to a mixture containing ATPase buffer (see 2.6.11.2.), 300 mM NaCl-elution buffer (see 2.6.7.) and radioactive ATP solution, thus starting the assay. After 0, 1, 5, 10 and 20 min samples were taken and the reaction stopped in 0.3 M perchloric acid/1 mM NaH_2PO_4. To remove the nucleotides the samples were mixed with charcoal. The samples were centrifuged for 1 min and 100 μl of the supernatant containing the free γ-^{32}P was transferred into a new Eppendorf cup. The radiation of the supernatants and the standard (1 mM radioactive ATP) was quantified in a scintillation counter (Canberra Packard, Frankfurt am Main) in the Cerenkov-mode. For determination of the basal activity the linear part of the reaction was used.

2.6.13.2. Coupled ATPase assay

Microtubules were polymerised as described in section 2.6.5.. The microtubule concentration was determined as in section 2.6.6..

In this test the ATP turnover of the kinesin constructs is measured in the steady state. The turnover of ATP by the kinesin is coupled by the enzymes pyruvate kinase (PK) and lactate dehydrogenase (LDH) to the consumption of NADH ($NADH \Rightarrow NAD^+$). Phosphoenolpyruvate (PEP) is used as co-substrate of pyruvate kinase. The decrease of the concentration of NADH is monitored at A_{340} (Huang and Hackney, 1994).

The reaction is as follows: 4.0 μl ATP, 4.0 μl NADH, 4.0 μl PEP, 2.0 μl of enzyme mix (PK, LDH) and x μl microtubules were mixed and the volume adjusted to 79 μl with ATPase buffer. The reaction was started by addition of 1μl kinesin (1:1 - 1:50 diluted). The measurements were done in a 50 μl quartz-cuvette (Hellma) for 3 min at 340 nm in an

UVIKON 930 photometer (Kontron, Lohhof) measuring in time-drive mode. The maximum slope of the curve for the first minute was determined. The optimal amount of kinesin for the measurements was determined first. For the measurements the microtubule concentrations were varied and the ATP concentration held constant. For the analysis of the data and the calculation of the $K_{0.5}$ Mt and the V_{max} the KaleidaGraph programme (Macintosh) was used. The decrease in extinction was plotted against the microtubule concentration and the curve fitted against the Michaelis-Menten equation (B = background oxidation of NADH):

$$y = (B + V_{max} \cdot [Mt]) / ([Mt] + K_{0.5}Mt).$$

The programme calculates the V_{max} (maximal decrease in extinction; $V_{max} = \Delta E/min$) and the $K_{0.5}$Mt (half-maximal activation constant).
For the determination of the K_m(ATP) the ATP concentration was varied and the microtubule concentrations held constant at a saturating level. The assay was performed at 22°C.

Solutions:

ATPase buffer:	12 mM ACES·KOH, pH 6.8, 25 mM K·acetate, 2 mM Mg-aceteate, 0.5 mM EGTA·KOH
ATP (4°C):	10 mM ATP in ATPase buffer (from 0.1 M stock ATP in dH_2O, pH 7.0 1:10 diluted in ATPase buffer)
Enzyme mix (4°C):	20 μl lactate dehydrogenase (5.5 U/μl, glycerol solution, Roche), 20 μl pyruvate kinase (2 U/μl, glycerol solution, Roche), 60 μl ATPase buffer
PEP (4°C):	phosphoenolpyruvate (PEP, mono-potassium salt, Sigma), 4 mg/ml solution in ATPase buffer, pH to 7.0 with KOH
NADH (4°C):	Sigma, 2 mg vial, dissolve content in 640 μl ATPase buffer
Microtubule dilution buffer:	ATPase buffer supplemented with 20 μM taxol
Kinesin dilution buffer:	300 mM NaCl-elution buffer (see 2.6.7.), 1 mg/ml BSA

2.6.13.3. Calculations for the ATPase assay

ATP consumption and NADH oxidation are coupled 1:1 in the assay. Therefore, the decrease of the extinction at 340 nm is a stoichiometric parameter for the ATP hydrolysis. The molar decrease of the ATP concentration can be calculated after the Lambert-Beer law:

$A = \varepsilon \cdot c \cdot d$ with

A	absorbance
ε	extinction coefficient (for NADH: $\varepsilon_{340} = 6.22 \cdot 10^3 \cdot M^{-1} \cdot cm^{-1}$)
c	concentration of the light absorbing substance
d	length of the light path (thickness of the cuvette, 1 cm)

The molar ATP turnover is calculated from the rearranged Lambert-Beer equation as follows:

$\Delta c/\Delta t = \Delta A/(\varepsilon \cdot d)$ with $\Delta A = V_{max}$ (based on the measured time, e.g. 1 min = 60 sec) .

(1) $\Delta c/\Delta t = V_{max} / (60 \cdot 6.22 \cdot 10^3)$ $[M \cdot sec^{-1}]$

The concentration of kinesin in the assay is calculated from the used volume and the protein concentration:

(2) $c_{kinesin} = (c_{protein} / Mr) \cdot (vol / 80\ \mu l)$ [M] with

$c_{kinesin}$	concentration of kinesin [M]
$c_{protein}$	protein concentration of the kinesin preparation [mg/ml]
Mr	molecular weight of the kinesin [Da]
vol	volume of the kinesin solution [μl] / 80 μl assay

The k_{cat} (maximal ATP turnover) is calculated by dividing (1) / (2) or $k_{cat} = (\Delta c/\Delta t) / c_{kinesin}$ $[sec^{-1}]$.

2.6.14. Antibodies

2.6.14.1. Immunization and preparation of antiserum

For antibody preparation the FPLC-purified NcKIF1 399 (see 2.5.17.1. and 2.5.19.) was used. A sample with a concentration of 3.62 +/- 0.33 mg/ml was send to the Pineda Antikörper-Service (Berlin, Germany), and antibody preparation was carried out there. Prior to immunization sera of 5 rabbits were tested against *N. crassa* and *E. coli* extracts for background exclusion or cross reactivity prior to immunization. Two rabbits were selected and the sera tested from day 1, 70 and 100 of immunization against the antigen preparation by Western blotting. The serum with the best signal (rabbit 2, 70th day of immunization) was used for affinity purification.

Preparation of *E. coli* extract: 1 g of the *E. coli*-strain BL 21 after expression of *N. crassa* NKin full length was solved in dH_2O very quickly on ice. A sample was taken, 1/5 volume of 6 x Laemmli sample buffer added, heated for 5 min at 95°C , centrifuged for 5 min at maximum speed in an Eppendorf centrifuge and the supernatant loaded on a PAA-gel.

Preparation of *N. crassa* extract: *N. crassa* wild type was taken from a liquid culture (see 2.4.2.) and prepared as described in section 2.6.15. (first method). The S2 was mixed with 1/5 volume of 6 x Laemmli sample buffer, heated for 10 min at 95°C and loaded on a PAA-gel.

2.6.14.2. Detection of proteins by Western blotting

Western blotting was carried out according to Sambrook et al., 1989. Proteins were separated by SDS-PAGE and transferred to a nitrocellulose membrane (Schleicher and Schuell, Protran® Nitrocellulose Transfer Membrane, pore size 0.45 μm). The transfer was carried out for 90 min and 0.8 mA/cm^2 in a semi-dry blotting chamber. The transferred proteins were stained with Ponceau S and the position of the standard proteins marked with a pencil. The membrane was blocked in 10 % milk powder in TBST for 1 h at room temperature or at 4°C over night, washed with TBST and incubated with the primary antibody (antibody in TBST + 1 % (w/v) BSA + 0.1 % Na-azide) for 1 - 2 h at room temperature or over night at 4°C. The membrane was now washed 1 x 15 min and 2 x 5 min in TBST and then incubated with the secondary antibody (goat-anti-rabbit IgG, whole molecule, peroxidase conjugate, Sigma, 1:10000 in TBST + 5 % milk powder) for 1 h at room temperature and following that washed again 1 x 15 min and 4 x 5 min. For detection, the chemiluminescence-based ECL-system (Amersham) was used. The Lumi-Light^{Plus} Western Blotting Substrate (Roche) was also used for high sensitivity detection. Detection was carried out according to the instructions of the manufacturer, using an X-ray film (Kodak) to visualize signals.

Transfer buffer: 25 mM Tris, 129 mM glycine, 0.0366 % SDS, 10 % methanol

Ponceau S-solution: 0.25 % Ponceau S, 40 % methanol, 15 % acetic acid

TBST: 20 mM Tris · HCl, pH 7.2, 150 mM NaCl, 0.05 % Tween 20

2.6.14.3. Affinity purification with nitrocellulose strips

FPLC-purified NcKIF1 399 (see 2.6.12.1.) was separated in a PAA-gel and transferred to nitrocellulose as described in 2.6.12.2.. The kinesin band was visualized with Ponceau S, cut out and washed 3 x 5 min in TBST. The strip was blocked in 5 % milk powder in TBST and washed 3 x 5 min in TBST. The antiserum was diluted 1:100 in TBST. The strip was cut into about 1 cm pieces and incubated with the antiserum for 1 - 2 h at room temperature in an Eppendorf cup under rotating. The stripes were washed 3 x 5 min in TBST. For elution of the antibody, 500 μl of 200 mM glycine/HCl, pH 2.8, was pipetted for 2 min over the pieces. The solution was neutralized with 13 μl of 2 M Tris and the pH checked with pH paper (Merck). The purified antibody solution was supplemented with glycerol 1:1 and stored at -20°C.

2.6.14.4. Dot blot test for determination of antibody sensitivity

FPLC-purified kinesin (NcKIF1 434) was diluted stepwise in 300 mM NaCl-elution buffer (see 2.6.7.) and 1 µl of each dilution was placed directly onto a nitrocellulose membrane. The following treatment was the same as described in section 2.6.12.2..

2.6.15. Preparation of *N. crassa* crude extract

N. crassa mycelium was grown for 16 h in a shaking culture and in a 4 l - culture under constant aeration as described in section 2.4.2., then harvested and weighed. Mycelium, sea sand and AP-100 supplemented with 50 µl of Pi, 20 µl of Pe and 5 µl of DTT (see 2.6.7.) for 5 ml of AP-100 were mixed at equal amounts at 4°C. The mycelium was ground for 5 – 15 min, and then centrifugation was carried out (20 min, 4°C, 12000 rpm, Sorvall rotor SS 34). The supernatant was centrifuged again (60 min, 4°C, 45000 rpm, Beckman rotor TI 70.1). A sample of the middle layer, named S2, was taken with a glass pipette and splitted into two parts. To one part 1/5 volume of 6 x Laemmli sample buffer was added for loading on a PAA-gel and heated for 10 min at 95°C prior to use. The other sample was used for the Bradford assay for determination of protein concentration (see 2.6.3.). The other layers (top: fatty layer, above bottom: vacuole-rich layer) as well as the pellet and the supernatant after the first centrifugation step (S1) were also examined. The pellet was taken out, 1 x Laemmli sample buffer added and heated for 10 min at 95°C prior to use. For dissolving membranes 1 % Triton X-100 (Sigma) was added to the sample after the first centrifugation.
In a second method after grinding centrifugation was carried out 2 x 10 min and 2 x 15 min at 4°C, 12000 rpm, Sorvall rotor SS 34. With this method, the crude extract corresponds more a cell extract, whereas with the first method the crude extract (S2) corresponds more a cytosolic extract. The supernatants were examined, a pellet was besides the sand not present.

2.6.16. Microtubule affinity enrichment from *N. crassa* cude extract

Preparation of *N. crassa* crude extract was carried out as described in 2.6.13.. Both types of extracts were generated. With the first method, the S2 and with the second method the cell extract after the fourth centrifugation were taken for microtubule affinity enrichment.
Microtubules were polymerised 20 min before centrifugation was finished. 0.5 mg tubulin/ml crude extract were used and polymerised with 1 mM GTP for 20 min at 37°C in a waterbath. 10 µM taxol were added to the microtubules. The crude extract was measured and transferred into a precooled Erlenmeyer flask. 10 µM taxol, 200 µM AMP-PNP, 5 U/ml apyrase and the microtubule-taxol mixture were added and mixed thoroughly. Incubation was performed for

1 h under shaking and after that, centrifugation was carried out (30 min, 4°C, 42000 rpm, Beckman rotor 42.1) to pellet the microtubules with the kinesins attached. The pellet was resuspended in 1 ml of KCl-buffer and centrifuged through a 1 ml sucrose cushion (10 min, 4°C, 80000 rpm, rotor TLA 100.3). The pellet was resuspended in 150 - 400 μl of release buffer and incubated for 15 min on ice. The microtubules were separated by centrifugation (10 min, 4°C, 80000 rpm, rotor TLA 100.3) and the supernatant with the released kinesins taken for analysis. The whole procedure was carried out at 4°C.

KCl-buffer: AP-100, 50 mM KCl, 10 μM taxol, 200 μM AMP-PNP

Sucrose cushion: 20 % sucrose in AP-100, 10 μM taxol

Release-buffer: AP-100, 10 mM ATP, 10 mM $MgCl_2$, 200 mM KCl, 10 μM taxol

3. RESULTS

3.1. Sequence of NcKIF1

3.1.1. Sequence of the NcKIF1 gene

Starting point were the λ10 and λ11/2 vectors which contain the NcKIF1 sequence found in a mycelial cDNA library of *Neurospora crassa* (FGSC; Seiler, 1999). Sequencing of the inserted cDNA showed a sequence which was homologous to other members of the UNC-104/KIF1 family of kinesin-like proteins in the motor domain. The tail domain exhibited no significant similarity to any other known protein sequences. A sequence alignment with other members of this family showed that the NcKIF1 sequence was not complete in the N-terminal part. Therefore, and also to confirm the unusual sequence in the C-terminal part, a different cDNA library (the mycelial cDNA library M-1; FGSC, the University of Kansas Medical Center, USA) was screened. A mycelial cDNA library was chosen again, because the sequence of NcKIF1 was first found in a mycelial cDNA library.

The screen was performed with a probe against the coding region of the motor domain and the following 14 amino acids of NcKIF1. The isolated cDNA had a size of 2510 bp and accommodated the coding sequence of NcKIF1 (1947 bp; Fig.1). The cDNA clone contained an ATG codon that extended the known sequence by 75 bp (corresponding to 25 amino acids) to the 5'-end, suggesting that it serves as the start codon. Besides the missing sequence at the N-terminus, the sequences in the λ10 and λ11/2 vectors were found to be identical to the coding sequence of the newly isolated cDNA of NcKIF1. The unusual sequence in the tail of NcKIF1 was confirmed.

The gene sequence of NcKIF1 contains an intron of 76 bp between bp 156 and bp 157 in the beginning of the coding sequence (Fig.2). This sequence can be found in the genomic DNA of *N. crassa* but not in the cDNA clones. The genomic sequence of NcKIF1 is contained in the *N. crassa* Genome Database of the Whitehead Institute Center for Genome Research, USA (contig 3.201). Sequencing of the cosmid clones X2G12 and G2B8 (cosmid library pMOcosX; Orbach, 1994; FGSC; Seiler, 1999) which represent genomic DNA of *N. crassa* showed this intron of 76 bp also to be present.

On the next two pages, Figure 1 shows the isolated cDNA sequence of NcKIF1. In Figure 2 the sequence of the intron in the gene sequence of NcKIF1 is depicted.

```
1     CAACACCATT GTGGATATCG AAGATACTTT ATACGACATA TCTTTGGAGC
51    CGCGCCTCAT GCCGAACTCC CTCGACGTCC ACCAGCGGCA GACCCGCTCC
101   AATGTCTCGA CTCCGACTTT GCGTCCTCGA GATGATACGG CTTCGTCTTT
151   CGTTTCCAAG GATCCCGGGG CAAATGTTCG GGTGGTGGTG AGAGTAAGAG
201   CATTTTTGCC TCGCGAACTC GAGCGCAATG CTGAATGCAT TGTTGAGATG
251   GACCCGGCAA CAGAACGAAC ATCCCTTCTG GTTCCCCAGG AGACAGACTT
301   CGCTGATGCT CGAGGTGCCC GGTCTCGCAG GGTACTGGAG GAGAAGTCGT
351   TTACCTTTGA TAAGAGCTTC TGGAGTCATA ATACAGAAGA CGAGCACTAC
401   GCGACACAGG AGCATGTCTA CGACAGCTTG GGCGAGGAAT TTCTCGATCA
451   CAACTTCGAA GGATACCACA CCTGTATCTT TGCCTACGGT CAGACTGGCT
501   CGGGGAAGTC TTATACCATG ATGGGAACGC CCGATCAACC CGGACTTATC
551   CCCAGAACTT GTGAAGATCT GTTCCAGCGC ATTGCTTCCG CCCAGGACGA
601   GACGCCCAAT ATCAGCTATA ATGTCAAAGT CAGCTATTTC GAAGTTTACA
651   ATGAACATGT GCGAGACCTT CTCGCTCCTG TCGTGCCCAA CAAGCCGCCA
701   TACTACCTCA AAGTCCGCGA ATCTCCTACC GAGGGTCCAT ATGTCAAAGA
751   CCTGACCGAG GTTCCCGTGC GAGGTCTCGA AGAGATCATC AGGTGGATGC
801   GTATTGGCGA TGGAAGCCGC ACAGTAGCCA GCACCAAGAT GAACGACACC
851   AGTAGCCGCA GCCATGCCGT CTTTACTATT ATGCTCAAAC AGATCCACCA
901   CGACCTAGAG ACAGATGATA CTACAGAGCG CAGCAGTCGT ATCCGCCTTG
951   TCGACTTAGC TGGCAGCGAG CGAGCAAAGT CCACCGAGGC GACTGGCCAA
1001  CGCCTCCGTG AAGGGAGCAA TATCAACAAG TCCCTAACCA CTCTGGGGCG
1051  TGTCATCGCC GCACTTGCCG ATCCGAAGTC AAGCGCAAGC CGCCCTTCTT
1101  CTCCCGTAAA ATCCGGAAGA GGACGAACGC CAGGGCCAGC CAACTCCGTG
1151  GTCCCCTACC GTGACAGCGT TCTCACCTGG CTCCTCAAAG ATTCCCTTGG
1201  CGGCAACTCC AAAACCGCCA TGATAGCCTG CATCTCCCCC ACCGACTATG
1251  ACGAGACGCT CTCCACGCTC CGCTACGCCG ACCAAGCCAA GCGCATTCGT
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1301 ACCCGTGCCG TGGTCAATCA AGTCGATGGC GTCAGCGCCG CTGAGCGCGA

1351 CGCCCAGATT GCCGCCATGG CTGCCGAGAT CCGCCAGTTA CAGTTGGTTG

1401 TGAGCGACAG CCAAACCCGT GAAAAGAGCG CTCTCGACGC CGAACAGCAG

1451 CTCGAGGAAT ATCAAGCGCG TGTCCGAGGC CTGCAGCAGC TGATGGAGGA

1501 GAAGAGCCTG GTGGCCGAGG GCAAAATCCG GTCTCTACAG ACGGAAAATG

1551 AAGCGTTGCG GTTGCATCTC AAGTTGGCCC TGGAGAGTCT TCGCAACCCT

1601 ATCAAGGTGT CTTCATTCCC CACTACCTCG CTGGCCATGT CCGCCGGGGA

1651 CTCGACCGTG CCGCTGATGG CCATGGGTGA GGTTGGTACC GCCCACGGCC

1701 GAAAGATGGA AAATAAGTTG GTGGACGACC CCTTCGTCGA TAGCGGCAGT

1751 GGCGTCACTG GTGATGACGA TCAAGGCCTC ATTTGTGGAC ATGATGATGA

1801 TTATGACACT TACGAAGAAG AAGACGACGA CGACGAAGAT ACAATTGATC

1851 TCAGCGAAAA GGCTCATGAC ATGAACGAGT ATATGAGTGG TTTGCTCAAG

1901 GATTTGAGCA TGTTTAGACG CAAGATCGGG GATGATAAGA CGAGGTTTCT

1951 GGATGAGTTG GGCGTCAGGA AACCGCTGGG GGTGAGGACT AATATCATGT

2001 GATGACGAAG TTGGTCTGGA TTCGACGTGT TTTGGTGAAC GGATTGATGG

2051 GATAACGCAA GCAAGCCAGG AAGGAGTTTA CGGACGGCGT TTTACAAAGG

2101 TTGAACGTAT GAGTCAACGA GTTTGGTCCT GGTGGCGTGG ATCTATAGCT

2151 TGAGGAAAAT GAGAAGGGCT TTACATGGAA GACGCAAAGG ATAGGAAAGG

2201 GAAGGAAAGC AGGTAAAAGC CATGGCGATG GGTGGAGTTT TGGCCTGGAC

2251 AAGGTGCGGC GGTAGGCATT TGCTTTCAAC TTCACATTTT CTTTCTCTCA

2301 TCGTTGTCTT CATTATACAC CGGAGTTATG TACAAGTTCT GGCTCGGCGT

2351 GGTTTCGGTT TCTCTTTGGC AATATCTGTA TTTCTTAAAC TTCTATTCCC

2401 CGGGTATATC GTTTTTCTGC GGTCAGTATG GGGCATAACA AGCATAATGC

2451 TCCATCAGGG ACTTTTAAGG TCTAAATCCA TCAATGGAAC TTGATTGACA

2501 TCCGAAAAAA

Fig.1. Sequence of the cDNA of NcKIF1. The coding sequence is underlined.

```
1     GGTAAGTTGG AAATCCATGT GTTTATTCCA CCACCAGACA CTGCTCACTC
51    CTATGCTAAC ACGTTTGCTT ATCATA
```

Fig.2. Sequence of the intron in the NcKIF1 gene. The intron is inserted between bp 156 and 157 of the coding sequence.

3.1.2. Protein sequence of NcKIF1

The DNA sequence of NcKIF1 codes for a protein of 647 amino acids with a calculated molecular weight of 72 kDa (Wisconsin Package Version 10.2, Genetics Computer Group (GCG), Madison, Wisc.; programme PEPTIDESORT). The amino acid sequence of NcKIF1 is shown in Figure 3.

```
1    MPNSLDVHQR QTRSNVSTPT LRPRDDTASS FVSKDPGANV RVVVRVRAFL
51   PRELERNAEC IVEMDPATER TSLLVPQETD FADARGARSR RVLEEKSFTF
101  DKSFWSHNTE DEHYATQEHV YDSLGEEFLD HNFEGYHTCI FAYGQTGSGK
151  SYTMMGTPDQ PGLIPRTCED LFQRIASAQD ETPNISYNVK VSYFEVYNEH
201  VRDLLAPVVP NKPPYYLKVR ESPTEGPYVK DLTEVPVRGL EEIIRWMRIG
251  DGSRTVASTK MNDTSSRSHA VFTIMLKQIH HDLETDDTTE RSSRIRLVDL
301  AGSERAKSTE ATGQRLREGS NINKSLTTLG RVIAALADPK SSASRPSSPV
351  KSGRGRTPGP ANSVVPYRDS VLTWLLKDSL GGNSKTAMIA CISPTDYDET
401  LSTLRYADQA KRIRTRAVVN QVDGVSAAER DAQIAAMAAE IRQLQLVVSD
451  SQTREKSALD AEQQLEEYQA RVRGLQQLME EKSLVAEGKI RSLQTENEAL
501  RLHLKLALES LRNPIKVSSF PTTSLAMSAG DSTVPLMAMG EVGTAHGRKM
551  ENKLVDDPFV DSGSGVTGDD DQGLICGHDD DYDTYEEEDD DDEDTIDLSE
601  KAHDMNEYMS GLLKDLSMFR RKIGDDKTRF LDELGVRKPL GVRTNIM**
```

Fig.3. Amino acid sequence of NcKIF1.

The molecule is homologous in the motor domain (amino acids 37-420, see sequence alignment, Fig.4) to other known members of the UNC-104/KIF1 family, but there is no significant similiarity in the tail domain. The term tail domain is used for the entire sequence behind the conserved motor domain. As a special feature, NcKIF1 has a 35 amino acid extension at the N-terminus.

The tail domain of NcKIF1 (227 aa) is short in comparison to the motor domain (420 aa). The motor domain of NcKIF1 contains the structural elements present in the motor domain of other members of the UNC-104/KIF1 family. For comparison, NcKin is shown as a

representative of a conventional kinesin (Fig.4). The members of the UNC-104/KIF1 family have a lysine-rich loop (K-loop) in their motor domain. This loop is also present in NcKIF1, but differs from that of other members (Fig.4).

A comparison between the motor domains of NcKIF1 and other members of the UNC-104/KIF1 family was performed and is shown in Table 2. The core motor domain of NcKin was also included into the comparison (amino acids 1-332, Vale and Milligan, 2000; Kallipolitou et al., 2001). The table shows that the motor domain of NcKIF1 is highly conserved.

Member	UmKin3 (aa 1-368)	DdUnc104 (aa 1-362)	Ceunc104 (aa 1-354)	MmKIF1A (aa 1-361)	MmKIF1B (aa 1-355)
Similarity	63.9 %	63.5 %	66.2 %	66.3 %	66.9 %
Identity	54.6 %	56.0 %	57.6 %	58.4 %	57.4 %

Member	MmKIF1Bb (aa 1-361)	RnKIF1D (aa 1-354)	HsKIF1C (aa 1-355)	HsATSV (aa 1-361)	NcKin (aa 1-332)
Similarity	65.4 %	65.9 %	67.4 %	66.3 %	57.2 %
Identity	56.5 %	57.6 %	58.6 %	58.7 %	48.6 %

Table 2. Comparison of the motor domains between NcKIF1 (aa 1-420) and other known members of the UNC-104/KIF1 family of kinesin-like proteins. NcKin (aa 1-332, core motor domain) was also compared and shows a lower degree of similarity and identity in comparison to UNC-104/KIF1 family members. The analysis was performed using the GCG programme GAP (Wisconsin Package Version 10.2, Genetics Computer Group (GCG), Madison, Wisc.).

```
1
  NcKIF1  MPNSLDVHQR QTRSNVSTPT LRPRDDTASS FVSKDPGANV RVVVRVRAFL
  UmKin3  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~MADSGNI KVVVRCRPMN
DdUnc104  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~MNV QVAVRVRPFN
Ceunc104  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~MSSV KVAVRVRPFN
 MmKIF1A  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~MAGASV KVAVRVRPFN
 MmKIF1B  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~MSGASV KVAVRVRPFN
MmKIF1Bb  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~MSGASV KVAVRVRPFN
 RnKIF1D  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~MAGASV KVAVRVRPFN
 HsKIF1C  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~MAGASV KVAVRVRPFN
  HsATSV  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~MAGASV KVAVRVRPFN
NcKin332  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~MSSSANSI KVVARFRPQN
```

```
          51
  NcKIF1  PRELERNAEC IVEMDPATER TSLLVPQETD FADARGARSR RVLEEKSFTF
  UmKin3  SRERNRGASN LIEFV..DQH QLILSP..PN EADTKENSKA TKKKSMPFSF
DdUnc104  SREKERNAEL IVQM..NNKS TILTRPSALR ...ANPLAAP TADDEKSFSF
Ceunc104  QREISNTSKC VLQVNGNT.. TTINGHSINK EN........ .....FSFNF
 MmKIF1A  SREMSRDSKC IIQMSGST.. TTIVNPKQPK ET........ ....PKSFSF
 MmKIF1B  SRETSKESKC IIQMQGNS.. TSIINPKNPK EA........ ....PKSFSF
MmKIF1Bb  SRETSKESKC IIQMQGNS.. TSIINPKNPK EA........ ....PKSFSF
 RnKIF1D  ARETSQDAKC VVSMQGNT.. TSIINPKQSR MF........ ....LKA.SF
 HsKIF1C  ARETSQDAKC VVSMQGNT.. TSIINPKQSK DA........ ....PKSFTF
  HsATSV  SREMSRDSKC IIQMSGST.. TTIVNPKQPK ET........ ....PKSFSF
NcKin332  RVEIESGGQP IVTFQGPD.. TCTVD...SK EA........ ....QGSFTF

          101                                                P-loop
  NcKIF1  DKSFWSH.NT EDEHYATQEH VYDSLGEEFL DHNFEGYHTC IFAY***GQTGSG***
  UmKin3  DRAYDEHTEQ DD........ LFQYIGVELL QHAFNGFNTC VFAY***GQTGSG***
DdUnc104  DYSYWSYDS. NDPHFASQST VYNDLGKEVL KNAWDGFNCS IFAY***GQTGSG***
Ceunc104  DHSYWSF.AR NDPHFITQKQ VYEELGVEML EHAFEGYNVC IFAY***GQTGSG***
 MmKIF1A  DYSYWSHTSP EDINYASQKQ VYRDIGEEML QHAFEGYNVC IFAY***GQTGAG***
 MmKIF1B  DYSYWSHTSP EDPCFASQNR VYNDIGKEML LHAFEGYNVC IFAY***GQTGAG***
MmKIF1Bb  DYSYWSHTSP EDPCFASQNR VYNDIGKEML LHAFEGYNVC IFAY***GQTGAG***
 RnKIF1D  DYSYWSHTSV EDPQFASQQQ VYRDIGEEML LHAFEGYNVC IFAY***GQTGAG***
 HsKIF1C  DYSYWSHTST EDPQFASQQQ VYRDIGEEML LHAFEGYNVC IFAY***GQTGAG***
  HsATSV  DYSYWSHTSP EDINYASQKQ VYRDIGEEML QHAFEGYNVC IFAY***GQTGAG***
NcKin332  DRVFDMSCKQ SDIFDFSIKP TVDDI..... ...LNGYNGT VFAY***GQTGAG***

          151
  NcKIF1  ***KS***YTMMGT.. ..PDQPGLIP RTCEDLFQRI ASA.QDETPN ISYNVKVSYF
  UmKin3  ***KS***HSMVGYA. ...QAKGIIP LTCARLFEDI NQK.TAADPN LKISVEVSYI
DdUnc104  ***KS***YSMMGYG. ...EEKGIIP LICEELFQRI QSTPSNSNEQ TIYKTTVSYM
Ceunc104  ***KS***YTMMG.KA NDPDEMGIIP RLCNDLFARI DNN.NDK..D VQYSVEVSYM
 MmKIF1A  ***KS***YTMMG.K. QEKDQQGIIP QLCEDLFSRI NDT.TND..N MSYSVEVSYM
 MmKIF1B  ***KS***YTMMG.K. QEESQAVIIP QLCEELFEKI NDN.CNE..E MSYSVEVSYM
MmKIF1Bb  ***KS***YTMMG.K. QEESQAVIIP QLCEELFEKI NDN.CNE..E MSYSVEVSYM
 RnKIF1D  ***KS***YTMMG.R. QEPGQQGIVP QLCEDLFSRV NVN.QSA..Q LSYSVEVSYM
 HsKIF1C  ***KS***YTMMG.R. QEPGQQGIVP QLCEDLFSRV SEN.QSA..Q LSYSVEVSYM
  HsATSV  ***KS***YTMMG.K. QEKDQQGIIP QLCEDLFSRI NDT.TND..N MSYSVEVSYM
NcKin332  ***KS***YTMMGTSI DDPDGRGVIP RIVEQIFTSI LSS.A.A..N IEYTVRVSYM

          201
  NcKIF1  EVYNEHVRDL LAPVVPNKPP YYLKVRESPT EGPYVKDLTE VPVRGLEEII
  UmKin3  EIYNEKVRDL LNP....KNK GNLKVREHPS LGPYVEDLSK LVVASYPDIM
DdUnc104  EIYNEKVKDL LNP..NNNKT GGLKVRNNPS TGPYVEDLSK LAVKSFSEID
Ceunc104  EIYCERVKDL LNP....NSG GNLRVREHPL LGPYVDDLTK MAVCSYHDIC
 MmKIF1A  EIYCERVRDL LNP....KNK GNLRVREHPL LGPYVEDLSK LAVTSYNDIQ
 MmKIF1B  EIYCERVRDL LNP....KNK GNLRVREHPL LGPYVEDLSK LAVTSYTDIA
MmKIF1Bb  EIYCERVRDL LNP....KNK GNLRVREHPL LGPYVEDLSK LAVTSYTDIA
 RnKIF1D  EIYCERVRDL LNP....KSR GSLRVREHPI LGPYVQDLSK LAVTSYADIA
 HsKIF1C  EIYCERVRDL LNP....KSR GSLRVREHPI LGPYVQDLSK LAVTSYADIA
  HsATSV  EIYCERVRDL LNP....KNK GNLRVREHPL LGPYVEDLSK LAVTSYNDIQ
NcKin332  EIYMERIRDL LAP....QN. DNLPVHEEKN RGVYVKGLLE IYVSSVQEVY
```

```
          251                    switch I
  NcKIF1  RWMRIGDGSR TVASTKMNDT SSRSHAVFTI MLKQIHHDLE TDDTTERSSR
  UmKin3  NLMDEGNKAR TVAATNMNET SSRSHAVFTL VLTQKRFDVQ TKLEAEKVSR
DdUnc104  MLMDEGSKAR TVASTNMNAT SSRSHAVFTI VFTQSKIDKT RGTAIDRVSK
Ceunc104  NLMDEGNKAR TVAATNMNST SSRSHAVFTI VLTQKRHCAD SNLDTEKHSK
 MmKIF1A  DLMDSGNKPR TVAATNMNET SSRSHAVFNI IFTQKRHDAE TNITTEKVSK
 MmKIF1B  DLMDAGNKAR TVAATNMNET SSRSHAVFTI VFTQKKQDPE TNLSTEKVSK
MmKIF1Bb  DLMDAGNKAR TVAATNMNET SSRSHAVFTI VFTQKKQDPE TNLSTEKVSK
 RnKIF1D  DLMDCGNKAR TVAATNMNET SSRSHAVFTI VFTQRSHDQL TGLDSEKVSK
 HsKIF1C  DLMDCGNKAR TVAATNMNET SSRSHAVFTI VFTQRCHDQL TGLDSEKVSK
  HsATSV  DLMDSGNKAR TVAATNMNET SSRSHAVFNI IFTQKRHDAE TNITTEKVSK
NcKin332  EVMRRGGNAR AVAATNMNQE SSRSHSIFVI TITQK..NVE TG..SAKSGQ

          301 switch II
  NcKIF1  IRLVDLAGSE RAKSTEATGQ RLREGSNINK SLTTLGRVIA ALADPKSSAS
  UmKin3  ISMVDLAGSE RANSTGATGA RLKEGANINR SLTTLGKVIA ALAIASSAVE
DdUnc104  ISLVDLAGSE RANSTGATGV RLKEGANINK SLSTLGKVIS ALAE.....
Ceunc104  ISLVDLAGSE RANSTGAEGQ RLKEGANINK SLTTLGLVIS KLAE...ES
 MmKIF1A  ISLVDLAGSE RADSTGAKGT RLKEGANINK SLTTLGKVIS ALAEMDSGPN
 MmKIF1B  ISLVDLAGSE RADSTGAKGT RLKEGANINK SLTTLGKVIS ALAEV.....
MmKIF1Bb  ISLVDLAGSE RADSTGAKGT RLKEGANINK SLTTLGKVIS ALAEVDNCTS
 RnKIF1D  ISLVNLAGSE RADSSGARGM RLKEGANINK SLTTLGKVIS ALADL.....
 HsKIF1C  ISLVDLAGSE RADSSGARGM RLKEGANINK SLTTLGKVIS ALADM.....
  HsATSV  ISLVDLAGSE RADSTGAKGT RLKEGANINK SLTTLGKVIS ALAEMDSGPN
NcKin332  LFLVDLAGSE KVGKTGASGQ TLEEAKKINK SLSALGMVIN ALTDGKS...
                                                          ------
          351    K-loop          MT-binding site
  NcKIF1  RPSSPVKSGR GRTPGPANSV VPYRDSVLTW LLKDSLGGNS KTAMIACISP
  UmKin3  PVKGAKK... .PKTASLDSF VPYRDSVLTW LLKDSLGGNS KTAMIAAISP
DdUnc104  .NSTSKKA.. ........VF VPYRDSVLTY LLKETLGGNS KTIMIAAISP
Ceunc104  TKKKKSNK.. ........GV IPYRDSVLTW LLRENLGGNS KTAMLAALSP
 MmKIF1A  KNKKKKKT.. ........DF IPYRDSVLTW LLRENLGGNS RTAMVAALSP
 MmKIF1B  .SKKKKKT.. ........DF IPYRDSVLTW LLRENLGGNS RTAMVAALSP
MmKIF1Bb  KSKKKKKT.. ........DF IPYRDSVLTW LLRENLGGNS RTAMVAALSP
 RnKIF1D  .QSKKRKS.. ........DF IPYRDSVLTW LLKENLGGNS RTAMIAALSP
 HsKIF1C  .QSKKRKS.. ........DF IPYRDSVLTW LLKENLGGNS RTAMIAALSP
  HsATSV  KNKKKKKT.. ........DF IPYRDSVLTW LLRENLGGNS RTAMVAALSP
NcKin332  .......... ........SH VPYRDSKLTR ILQESLGGNS RTTLIINCSP
          -------------L12------------
          401
  NcKIF1  TD..YDETLS TLRYADQAKR IRTRAVVNQV DGVS.AAERD AQIAAMAAEI
  UmKin3  AD..YEETLS TLRYADQAKK IKNKAVVNED PNAKLIRELK EELELLRTRV
DdUnc104  ADINYEESLS TLRYADSAKK IKTVAVVNED AQSKLIRELQ GEVERLRAMM
Ceunc104  ADINFDETLS TLRYADRAKQ IVCQAVVNED PNAKLIRELN EEVIKLRHIL
 MmKIF1A  ADINYDETLS TLRYADRAKQ IRCNAIINED PNNKLIRELK DEVTRLRDLL
 MmKIF1B  ADINYDETLS TLRYADRAKQ IKCNAVINED PNAKLVRELK EEVTRLKDLL
MmKIF1Bb  ADINYDETLS TLRYADRAKQ IKCNAVINED PNAKLVRELK EEVTRLKDLL
 RnKIF1D  ADINYEETLS TLRYADRTKQ IRCNAVINED PNARLIRELQ EEVARLRELL
 HsKIF1C  ADINYEETLS TLRYADRTKQ IRCNAIINED PNARLIRELQ EEVARLRELL
  HsATSV  ADINYDETLS TLRYADRAKQ IRCNAVINED PNNKLIRELK DEVTRLRDLL
NcKin332  SSYNDAETLS TLRFGMRAKS IKN~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
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```
          451
  NcKIF1  RQLQLVVSDS QTREKSALDA EQQLEEYQAR VRGLQQLMEE KSLVAEGKIR
  UmKin3  SGGG...... .........G ADGESNWDPS IPPDKQVVRY QTKTGE....
DdUnc104  DQGGQYHAND SKLMNSDYDE TVSTLN.... .......... ..........
Ceunc104  KDKGI.DVTD VQE....... TPGKHKKGPK LPAH...... ..........
 MmKIF1A  YAQGLGDITD M......... .......... .......... .........T
 MmKIF1B  RAQGLG.... .......... .......... .........D IIDTSMGS..
MmKIF1Bb  RAQGLGDIID IDPLIDDYSG SGGKYLKDFQ NNKHRYLLAS ENQRPGNFST
 RnKIF1D  MAQGLS.... .......... .......... .....ASALG GLKVEEGSPG
 HsKIF1C  MAQGLS.... .......... .......... .....ASALE GLKTEEGSVR
  HsATSV  YAQGLGDITD M......... .......... .......... .........T
NcKin332  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~

          501
  NcKIF1  SLQTENEALR LHLKLALESL RNPIKVSSFP TTSLAMSAGD STVPLMAMGE
  UmKin3  .......... .......... .......... ...IKTVTKA ELQEQLEQSE
DdUnc104  .......... .......... .......... .......... ...EKIEQYE
Ceunc104  .......... .......... ........VH EQ........ ..LEKLQESE
 MmKIF1A  NALVGMSPSS SLSALSSRA. .AS..VSSLH ERILFAPGSE EAIERLKETE
 MmKIF1B  .....LTSSP SSCSLNSQVG LTS..VTSIQ ERIMSTPGGE EAIERLKESE
MmKIF1Bb  ASMGSLTSSP SSCSLNSQVG LTS..VTSIQ ERIMSTPGGE EAIERLKESE
 RnKIF1D  GVLPAASSPP APASPSSPPP HNGELEPSFS PSAEPQIGPE EAMERLQETE
 HsKIF1C  GALPAVSSPP APVSPSSPTT HNGELEPSFS PNTESQIGPE EAMERLQETE
  HsATSV  NALVGMSPSS SLSALSSRA. .AS..VSSLH ERILFAPGSE EAIERLKETE
NcKin332  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~

          551
  NcKIF1  VGTAH.GRKM ENKLVDDPFV DSGSGVTGDD DQGLICGHDD DYDTYEE.ED
  UmKin3  KIMSSLNESW EEKLTKTQEI QKEREK.ALE ELGISV.... DKGNVGVHTP
DdUnc104  KLMAELNKSW EEKLSEAEAI REDRMA.ALK DMGVAIK... ......VVS.
Ceunc104  KLMAEIGKTW EQKLIHTEEI RKQREEE.LR DMGLACAEDG T..TLGVFSP
 MmKIF1A  KIIAELNETW EEKLRRTEAI RMEREAL.LA EMGVAMREDG G..TLGVFSP
 MmKIF1B  KIIAELNETW EEKLRKTEAI RMEREAL.LA EMGVAIREDR G..DIGVFSP
MmKIF1Bb  KIIAELNETW EEKLRKTEAI RMEREAL.LA EMGVAIREDG G..TLGVFSP
 RnKIF1D  KIIAELNETW EEKLRKTEAL RMEREAL.LA EMG....SPG GWRTVGVFSP
 HsKIF1C  KIIAELNETW EEKLRKTEAL RMEREAL.LA EMGVAVREDG G..TVGVFSP
  HsATSV  KIIAELNETW EEKLRRTEAI RMEREAL.LA EMGVAMREDG G..TLGVFSP
NcKin332  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~

          601
  NcKIF1  DDDEDTIDLS EKAHDMNEYM SGLLKDLSMF RRKIGDDKTR FLDELGVRKP
  UmKin3  KKLPHLVNLN EDPL.MSECL IYQIK...PG HTLVGNLDSG PDVHIKLSGT
DdUnc104  .SIPHLINLN EDPL.MSESL IYYVKE...G KTRIGRSDSE IPQDIILNGL
Ceunc104  KKLPHLVNLN EDPL.MSECL IYYLKE...G VTSVGRPEAE HRPDILLSGE
 MmKIF1A  KKTPHLVNLN EDPL.MSECL LYYIKD...G VTRVGREDAE RRQDIVLSGH
 MmKIF1B  KKTPHLVNLN EDPL.MSECL LYYIKD...G ITRVGQADAE RRQDIVLSGA
MmKIF1Bb  KKTPHLVNLN EDPL.MSECL LYYIKD...G ITRVGQADAE RRQDIVLSGA
 RnKIF1D  KKTPHLVNLN EDPL.MSECL LYHIKD...G VTRVGQVDV. ...DIKLTGQ
 HsKIF1C  KKTPHLVNLN EDPL.MSECL LYHIKD...G VTRVGQVDM. ...DIKLTGQ
  HsATSV  KKTPHLVNLN EDPL.MSECL LYYIKD...G ITRVGREDGE RRQDIVLSGH
NcKin332  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
```

```
          651
  NcKIF1  LGVRTNIM** ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
  UmKin3  KILNKHCMF. .......... .......... DHQDGLVTVT A.........
DdUnc104  NIHKEHCIFE NINGKVIISP SNNFMNNNNN KENSSSTTPT SSKSPSKPKS
Ceunc104  AILELHCEF. .......... .......INE D...GNVTLT M.........
 MmKIF1A  FIKEEHCIF. .......... .......RSD SRGGGEAVVT L.........
 MmKIF1B  HIKEEHCLF. .......... .......RSE RSNTGEVIVT L.........
MmKIF1Bb  HIKEEHCLF. .......... .......RSE RSNTGEVIVT L.........
 RnKIF1D  FIREQHCLF. .......... .......RSI PQPDGEVMVT L.........
 HsKIF1C  FIREQHCLF. .......... .......RSI PQPDGEVVVT L.........
  HsATSV  FIKEEHCVF. .......... .......RSD SRGGSEAVVT L.........
NcKin332  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~

          701
  NcKIF1  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
  UmKin3  .......... ....MP..DS MTMVNGKRLA PDEPKRLRSG YRVILGDFHV
DdUnc104  EKEKENNNDD DDGEKKLDRS YIYVNG..VE INKPTILTTG NRVILGNNHI
Ceunc104  .......... ....KP..NA SCYINGKQVT ..TPTVLHTG SRVILGEHHV
 MmKIF1A  .......... ....EPCEGA DTYVNGKKVT ..EPSILRSG NRIIMGKSHV
 MmKIF1B  .......... ....EPCERS ETYVNGKRVA ..HPVQLRSG NRIIMGKNHV
MmKIF1Bb  .......... ....EPCERS ETYVNGKRVA ..HPVQLRSG NRIIMGKNHV
 RnKIF1D  .......... ....EPCEGA ETYVNGKLVT ..EPLVLKSG NRIVMGKNHV
 HsKIF1C  .......... ....EPCEGA ETYVNGKLVT ..EPLVLKSG NRIVMGKNHV
  HsATSV  .......... ....EPCEGA DTYVNGKKVT ..EPSILRSG NRIIMGKSHV
NcKin332  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
```

Fig.4. Sequence alignment of NcKIF1 with sequences of other members of the UNC-104/KIF1 family of kinesin-like proteins. The 35 amino acid extension is depicted in bold letters and underlined. The end of the conserved motor domain is indicated by a frame (last amino acid; (*) Okada et al., 1995). NcKin332 represents the core motor domain of NcKin, a conventional kinesin (Vale and Milligan, 2000; Kallipolitou et al., 2001). The last amino acid of NcKIF1 434 as well as the beginning of NcKIF1 399 (second amino acid, the first is the methionine) is marked with a black box. NcKIF1 399 ends with the same amino acid as NcKIF1 434. The conserved P-loop, switch I and switch II motifs and the microtubule-binding site in the motor domain are indicated in bold and italic letters. The K-loop is underlined and positively charged residues are depicted in bold letters. The beginning and the end of the K-loop were selected according to Kikkawa et al., 2000. The K-loop is an amino acid insertion in loop 12 (L12) of the KIF1 motor domain. The complete sequences are shown in the Appendix.

The alignment was performed using the GCG programme PILEUP (Wisconsin Package Version 10.2, Genetics Computer Group (GCG), Madison, Wisc.). Sequences: NcKIF1-*Neurospora crassa*, NcKIF1; UmKin3-*Ustilago maydis*, unc-104 homologue (sequence from Wedlich-Söldner, R. and Steinberg, G.; Wedlich-Söldner et al., 2002); DdUnc104-*Dictyostelium discoideum*, unc-104 homologue (GenBank accession number AF245277); Ceunc104-*Caenorrhabditis elegans* kinesin-related protein unc-104 (M58582); MmKIF1A-*Mus musculus*, KIF1A (D29951); MmKIF1B-*Mus musculus*, KIF1B (D17577); MmKIF1Bb-*Mus musculus*, KIF1B major isoform (KIF1Bb) (AF131865); RnKIF1D-*Rattus norvegicus*, KIF1D (AJ000696); HsKIF1C-*Homo sapiens*, KIF1C (AB014606); HsATSV-*Homo sapiens*, axonal transporter of synaptic vesicles (X90840); NcKin-*Neurospora crassa* conventional kinesin (L47106).

3.2. Expression and purification

To examine whether NcKIF1 can be bacterially expressed and purified resulting in an active protein, the sequence coding for the motor domain and the following 14 amino acids from the λ10 vector was cloned into the pT7-7 vector (NcKIF1 399). The λ10 vector contained the first available sequence of NcKIF1, and the motor domain was cloned for using the protein to raise an antibody against the motor domain of NcKIF1.
The *E. coli* strain BL 21 was transformed with the plasmid pT7-7, containing the NcKIF1 399 sequence, and the protein expressed with 500 μM IPTG for 3 h and 23 h at 22°C and 37°C. Western blot and SDS-Page analysis showed an expression under these conditions. The kinesin was detected in Western blots using an antibody against the conserved motif „VDLAGSE“ in the kinesin motor domain, MMR44 (Marks et al., 1994).
To test the solubility of the protein, NcKIF1 399 was expressed in the *E. coli* strain BL 21 and induced with 500 μM IPTG for 23 h at 22°C and 37°C. A soluble, highly expressed protein was seen using an expression temperature of 22°C. Furthermore, two different IPTG concentrations (100 μM and 500 μM) were tested to determine whether the concentration of IPTG has an influence on the expression of the protein. A slightly higher expression was seen with 500 μM IPTG, which was therefore used from now on. Further studies on NcKIF1 434 showed that a shorter expression time had no effect on expression levels so that an expression time of 2-23 h could be used.
To test the purification conditions bacterially expressed NcKIF1 399 was prepared as described in section 2.6.7.2.. Anion- (DEAE-sephadex column) and cation exchange chromatography (SP-sepharose column) were performed and the flow-through as well as the elution fractions tested for activity in the ATPase assay. The results showed that the protein eluted at 300 mM NaCl from the cation exchange column. Therefore, an S-sepharose column was used for further purification.

To study the basic properties of NcKIF, a short construct was generated containing the conserved motor domain of NcKIF1 and the following 14 amino acids (Fig.4). This protein, NcKIF1 434, is 434 amino acids long and has a predicted size of 48.5 kDa [4)]. NcKIF1 434 expressed well in *E. coli*. Purification was performed with the FPLC using an S-sepharose column as described above. The protein eluted in the 300 mM NaCl-fraction (Fig.5, left).

[4)] The calculation of the molecular weight was performed using the GCG programme PEPTIDESORT (Wisconsin Package Version 10.2, Genetics Computer Group (GCG), Madison, Wisc.).

The expression of the full-length protein, NcKIF1 647, in *E. coli* yielded a partially degraded protein (Fig.5, right). The amount of degradation was variable, but generally about 40 % of the protein was not degraded. Attachment of the kinesin to microtubules by AMP-PNP and subsequent release by ATP revealed the same pattern of the protein bands in the PAA-gel as in the bacterially expressed protein. This supports a C-terminal degradation of the protein. Furthermore, the degradation of the bacterially expressed NcKIF1 647-protein was confirmed by Western blotting using an affinity-purified antibody raised against the motor domain of NcKIF1.

To try to improve the expression pattern of NcKIF1 647, expression studies were performed using different *E. coli* strains. The strains BL 21, BL 21 RIL and MC1061 were tested. The bacteria were induced at standard conditions (see above). Expression times from 2 h to 16 h were selected. However, neither of the strains improved the expression pattern. The degradation of NcKIF1 647 was already evident after 2 h of induction.

For protein modifications a cys-tag was introduced at the C-terminus of NcKIF1 434 and NcKIF1 647. The cys-tag contains 9 amino acids (-PSIVHRKCF; Itakura et al., 1993) with a reactive cysteine residue at the penultimate position. This residue can be used to conjugate the protein with, for example, biotin (see 2.6.11.). No difference in the expression of the proteins with and without cys-tag could be observed.

All expressed proteins were active in the microtubule-stimulated ATPase assay (see 3.3.1.), indicating correct folding of the proteins.

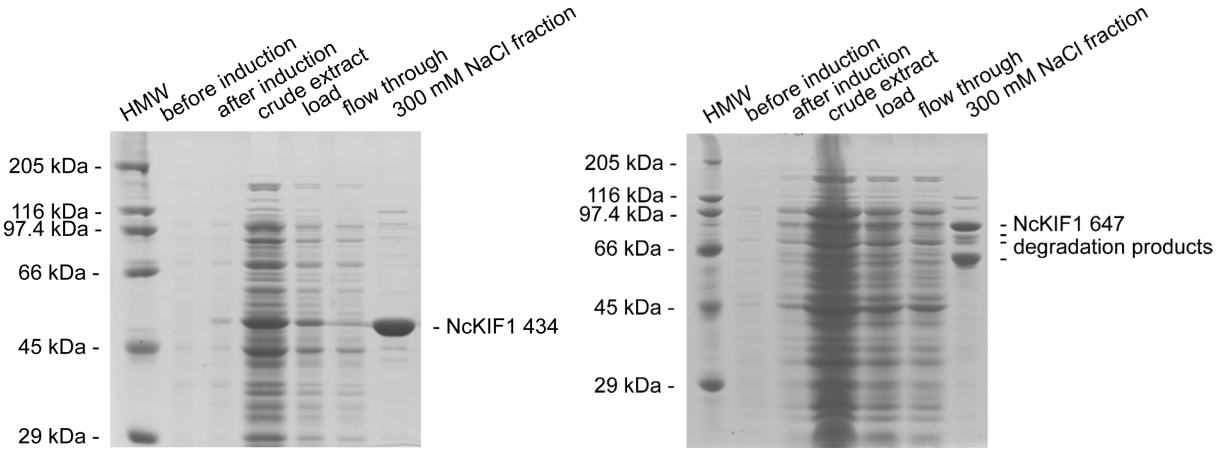


Fig.5. Left: Expression of NcKIF1 434 in *E.coli* and FPLC purification. NcKIF1 434 expressed well.
Right: Expression of NcKIF1 647 in *E.coli* and FPLC purification. Bacterially expressed NcKIF1 647 showed degradation products. The amount of degradation was variable dependent on the preparation. On PAA-gels the 60 kDa-band was seen as a double band in some preparations. Degradation was confirmed by Western blotting using an affinity-purified antibody raised against the motor domain of NcKIF1.

3.3. Biochemical studies of NcKIF1

3.3.1. Steady-state ATPase assay

To measure the microtubule-stimulated steady-state ATPase activity a coupled ATPase assay was used (2.6.13.2.). Two examples of ATPase assays are shown in Figure 6.

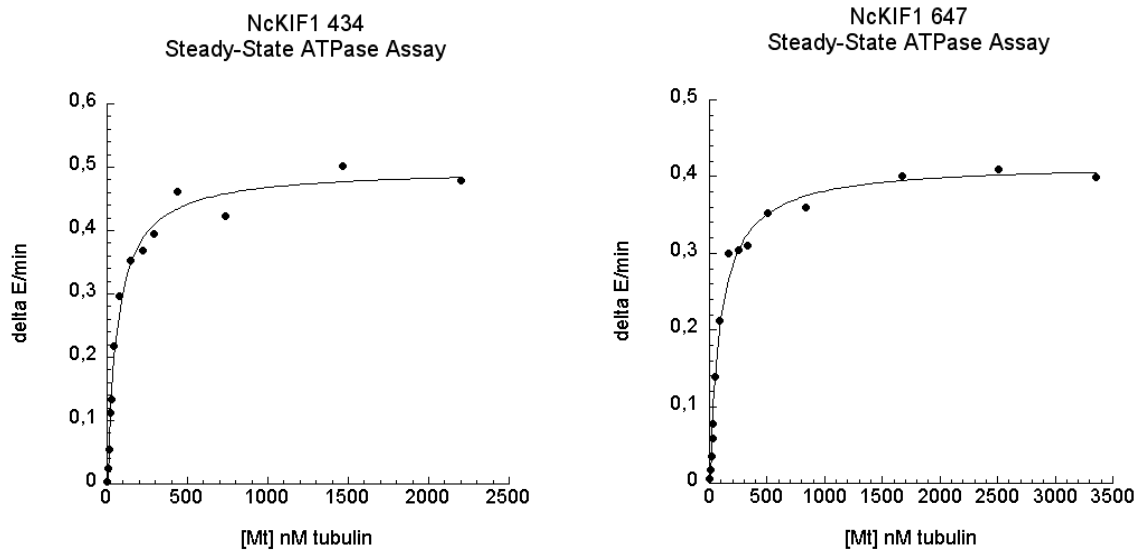


Fig.6. Microtubule-stimulated steady-state ATPase assay of NcKIF1 434 (left) and NcKIF1 647 (right). The curves follow a Michaelis-Menten kinetics. The microtubule-stimulated ATPase activity of NcKIF1 399 follows a Michaelis-Menten kinetics as well.

The following values were measured (Table 3):

Construct	k_{cat} [sec^{-1}]	$K_{0.5}$ Mt [nM tubulin]	Number of Preparations
NcKIF1 434	34 +/- 6	36.3 +/- 13.4	7
NcKIF1 647	15 +/- 3	108.7 +/- 27.3	5
NcKIF1 399	46	14.6 +/- 3.7	1

Table 3. Averaged k_{cat}- and $K_{0.5}$ Mt-values of the NcKIF1-constructs. The values are the mean and standard deviation of the indicated number of independent protein preparations.

The activity of NcKIF1 399 was measured just once to determine whether the expression results in an active protein.

There was no significant difference in the activity between preparations with and without DTT or the cys-tag. The expression of NcKIF1 647 resulted in a degraded protein. This has to

be considered during the evaluation of the data, because the length of the kinesin may have an influence on the values measured in the ATPase assay (Kallipolitou et al., 2001). The k_{cat}-values for NcKIF1 647 were calculated as described in 2.6.13.3. using the protein concentrations determined in the Bradford assay.

3.3.2. Basal ATPase activity

Kinesins are microtubule-activated ATPases. Without microtubules, they have a very low ATPase activity. This basal ATPase activity was measured for NcKIF1 434. For the full-length protein, NcKIF1 647, the activity was not measured because the bacterially expressed protein was degraded and no conclusions as to the activity of pure full-length protein can be drawn. Because the basal ATPase activity is very low, radioactive [γ-^{32}P]-ATP was used (2.6.13.1.).

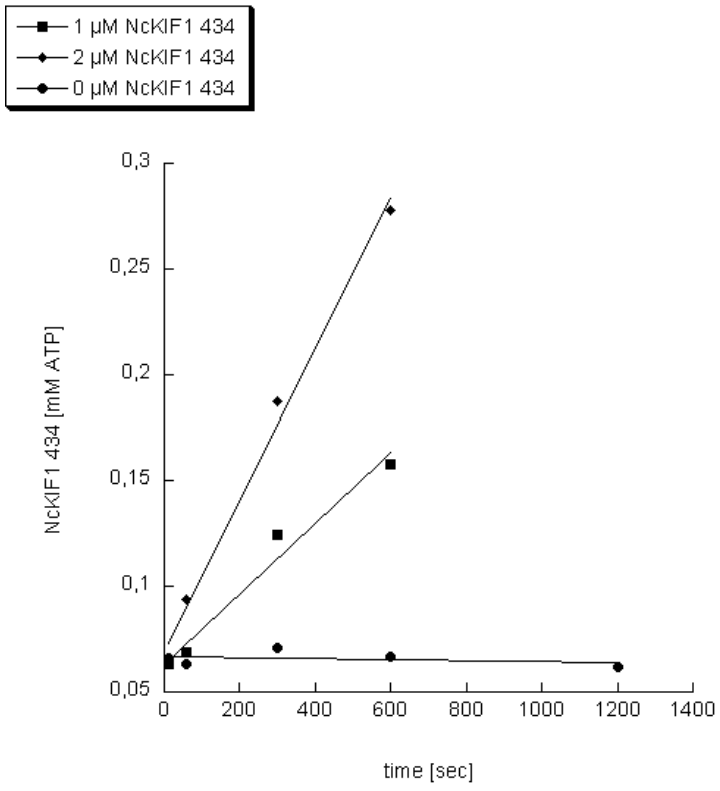


Fig.7. Basal ATPase activity of NcKIF1 434. The graph shows the emergence of free phosphate by NcKIF1 434 in the absence of microtubules. The slope of the free phosphate production corresponds to the rate of ATP turnover.

To determine the basal ATPase activity 1 μM and 2 μM NcKIF1 434 were mixed with 2 mM [γ-^{32}P]-ATP in the absence of microtubules at room temperature. The reaction was stopped at different times with perchloric acid and the free [γ-^{32}P]-phosphate of the supernatants was measured. The ATP consumption was determined on the basis of the radiation (cpm) of the

supernatants and the radiation (cpm) of a 1 mM [γ-^{32}P]-ATP standard. For the ATP standard double values were counted three times. The mean of the values was taken. By means of the programme KaleidaGraph a graph was drawn showing the calculated ATP consumption over time (Fig.7). The slopes of the curves (mM ATP/sec) were corrected with the blank value (measurement without kinesin; basal radiation). On the basis of the protein concentration in the assay and the corrected slopes of the curves the basal ATPase activity (k_0) was calculated. The basal ATPase activity was determined in two independent protein preparations of NcKIF1 434. The values for 1 μM and 2 μM kinesin were averaged.

For NcKIF1 434 a basal ATPase activity (k_0) of 0.144 +/- 0.039 sec^{-1} (n=2) was measured.

With a k_{cat} of 34 +/- 6 sec^{-1} of NcKIF1 434 in the microtubule-stimulated steady-state ATPase assay (3.3.1.) and a k_0 of 0.144 +/- 0.039 sec^{-1}, the enzyme is activated 236-fold by microtubules.

3.3.3. Multiple motor gliding assay

To further investigate the in vitro behaviour of NcKIF1, the multiple motor gliding assay was performed. In this assay the kinesin molecules are attached to a glass surface. After the addition of microtubules and Mg^{2+} ATP the microtubules are moved over the glass surface by the kinesin molecules; this movement can be visualised by video-enhanced light microscopy (2.6.12.).

3.3.3.1. NEM-inhibition test and Cy3-labelling

Because NcKIF1 434 lacked the C-terminus, this construct did not bind to the coverslip by itself and therefore had to be biotinylated to attach it to a streptavidin-coated glass surface. For this, a cys-tag (amino acids PSIVHRKCF; Itakura S., 1993; reactive cysteine residue underlined) was introduced at the C-terminus of NcKIF1 434. The cys-tag contains a reactive cysteine residue which can be conjugated with biotin using biotin-maleimide (2.6.11.). A flow chamber was coated with streptavidin and then incubated with the biotinylated kinesin. Biotin binds to streptavidin, thus attaching the protein to the glass surface. Now, microtubules and substrate can be added to observe microtubule movements.
To perform the multiple motor gliding assay using the streptavidin-biotin system it had to be tested whether NcKIF1 434 contains any other reactive cysteine residues besides the one introduced with the cys-tag. This is important to know because other reactive cysteine

residues could also be coupled with biotin, thus binding the protein to the glass surface in an unwanted manner and therefore interfering with the gliding assay. NcKIF1 434 has 4 cysteines in its amino acid sequence.

As a first test, an NEM-inhibition assay was performed. NEM interacts with reactive cysteine residues of a protein and usually leads to its inactivation. The DTT from the protein preparation was removed by gel filtration before the addition of NEM (2.6.9.).

In this test, NcKIF1 434 was incubated with NEM (about 40-fold molar excess) on ice for 5 min, 15 min, 30 min and 1 h. After these times the activity of NcKIF1 434 was measured in the ATPase assay. As a control, 1 mM NEM was inactivated with 10 mM DTT and added to the kinesin as above. The protein was incubated on ice for 30 min and 1 h and the activity of NcKIF1 434 measured in the ATPase assay. The data were evaluated using the KaleidaGraph programme (Fig.8.).

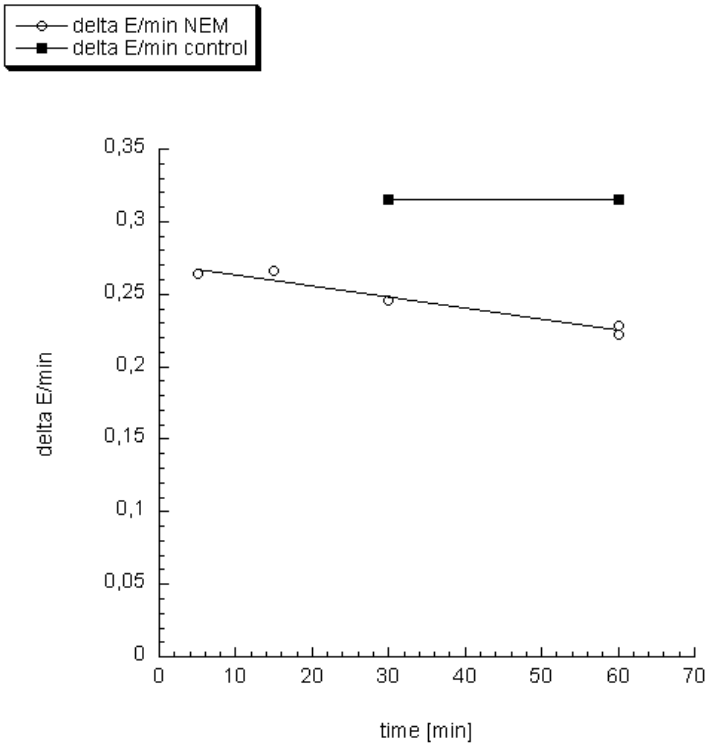


Fig.8. NEM-inhibition test. A 40-fold molar excess of NEM was added to NcKIF1 434 and the activity measured in the ATPase assay after 5 min, 15 min, 30 min and 1 h (open circles). As a control, 1 mM NEM was inactivated with 10 mM DTT and added to the kinesin. The activity of the protein was measured after 30 min and 1 h (closed squares).

A slight decrease of the activity of NcKIF1 434 could be seen in comparison to the control as well as over the time. It has to be considered that NEM was added to the kinesin at an about 40-fold molar excess. Considering that, the slightly slower activity and decrease over the time

could be due to a more general effect of NEM. Therefore, it can be assumed that NcKIF1 434 does not contain a reactive cysteine at a physiologically sensitive position in its sequence.
To confirm that NcKIF1 434 does not contain a reactive cysteine, Cy3-labelling was performed. The number of bound Cy3-molecules per kinesin can then be determined. For Cy3-labelling NcKIF1 434cys (a DTT-free preparation) was used.
NcKIF1 434cys contains one reactive cysteine in the C-terminally introduced cys-tag. If there is no other reactive cysteine residue in the protein, NcKIF1 434cys should just be labelled with one Cy3-molecule. The labelling procedure was performed as described in section 2.6.10.. Two releases of the Cy3-labelled NcKIF1 434cys were performed and the A_{552} - absorption for the Cy3 concentration measured. The results clearly show that in the first release one Cy3-molecule has bound to one kinesin molecule. The calculations performed with the values for the second release support this result. Together with the results from the NEM-inhibition test it can be concluded that NcKIF1 434cys does not contain any other reactive cysteine residues besides that in the cys-tag.

3.3.3.2. Results of the multiple motor gliding assay

For NcKIF1 434cys the multiple motor gliding assay was performed using a biotin-streptavidin system. The protein was biotinylated at the C-terminally introduced cys-tag. A flow chamber was coated with streptavidin and the protein bound to streptavidin via the biotin. The short motor domain construct did not attach to the glass surface alone. The assay was performed at 23°C and at saturating conditions of ATP (10 mM ATP in the assay).

For the motor domain construct NcKIF1 434cys, a gliding velocity of 0.46 μm/sec +/- 0.06 μm/sec (n=2) was measured for two independent protein preparations using 100 mM KCl in the gliding assay.

NcKIF1 647 was also measured. In this case, a streptavidin-biotin system was also planned to remove the degradation products of the protein preparation. This unfortunately failed, because testing of a non-biotinylated protein preparation of NcKIF1 647 showed that the gliding assay worked without using the streptavidin-biotin system. Which proteins of the solution are attached to the glass surface can not be determined. Therefore, for the gliding velocity of NcKIF1 647 it has to be considered that the protein preparation was degraded and contains a mix of C-terminally shorter proteins which can also contribute to the gliding velocity in this assay. The gliding assay was performed under the same conditions as for NcKIF1 434cys

(23°C, 10 mM ATP and 100 mM KCl). A conventional gliding assay without a streptavidin-biotin attachment was used (2.6.12.).

For the NcKIF1 647 preparation a gliding velocity of 0.60 +/- 0.05 μm/sec (n=3) was measured for three independent preparations. Thus the gliding velocity for the NcKIF1 647 preparation was higher than the velocity measured for NcKIF1 434.

Next, the influence of the KCl concentration on the gliding velocity of NcKIF1 434cys was examined. The gliding velocities of NcKIF1 434cys were measured using 0 mM, 50 mM, 100 mM, 150 mM, 200 mM, 250 mM and 300 mM KCl in the assay. The assay was performed as described for NcKIF1 434cys above (streptavidin-biotin system, 23°C, 10 mM ATP) but using different KCl concentrations. For each KCl concentration a new flow chamber was used. The velocities of 20 microtubules were measured for each concentration and the mean calculated. The results are shown in Figure 9. Two independent protein preparations were measured.

It can be concluded that the KCl concentration has no influence on the gliding velocity of NcKIF1 434cys. At higher salt concentrations a decrease in the affinity of the microtubules to the kinesin could be observed. At 300 mM KCl in the assay no binding of the microtubules to the kinesin could be seen any more.

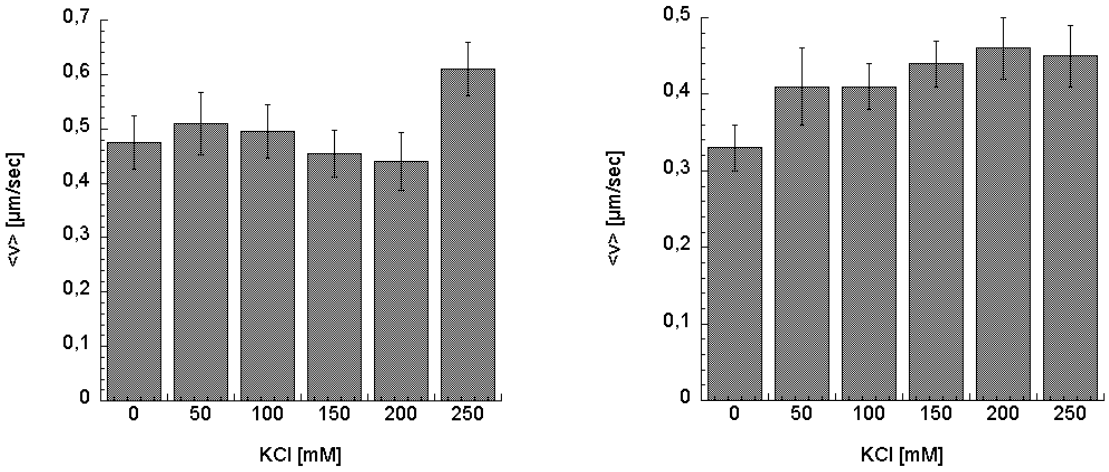


Fig.9. Multiple motor gliding assay with NcKIF1 434cys and different KCl concentrations. The velocities of 20 microtubules were measured. Two different protein preparations were measured independently. The velocities of the left graph were measured at room temperature. The velocities of the right graph were measured at 23°C. The high velocity of 0.61 μm/sec at 250 mM KCl in the left graph could not be confirmed in the second experiment. No significant influence of the KCl concentrations on the gliding velocity could be observed.

The K_m-values for ATP were determined for NcKIF1 434cys in the ATPase assay and in the multiple motor gliding assay. The ATP concentration was reduced stepwise and the microtubule concentration held constant. Finally no ATP was added. The measurements were performed with two independent protein preparations under the same conditions.
For the determination of the K_m-value for ATP the ATPase assay was performed as described in 2.6.13.2.. The ATP concentration was varied and the microtubule concentration held constant at a saturating level. For the analysis of the data, the programme KaleidaGraph was used. The plot was fitted against the Michaelis-Menten equation and the K_m-value for ATP calculated by the programme.

For the K_m(ATP), values of 42.8 μM ATP and 45 μM ATP were determined for NcKIF1 434cys in the ATPase assay.

In the multiple motor gliding assay the ATP was reduced starting with 2 mM ATP. The microtubule concentration and the other conditions were kept constant. The assay was performed at 23°C and a new flow chamber was used for each ATP concentration. To prolong the measuring time at low ATP concentrations a phosphoenolpyruvate / pyruvate kinase-regenerating system for ATP was used in the assay. Just the first minute of the recording was evaluated. The velocity of 20 microtubules was measured for each ATP concentration and the mean calculated. Below an ATP concentration of 0.1 mM in the assay less than 20 microtubules were measured (at 0.08 mM ATP 10 Mt, Fig.10, and with the second preparation 5 Mt at 0.08 mM ATP, 4 Mt at 0.05 mM ATP). This was caused by the low gliding velocities of the microtubules at these ATP concentrations, and that just the first minute of the recording was taken for the evaluation. At 0 mM ATP no movements of the microtubules could be observed anymore. The data were analysed as described above for the K_m(ATP)-values in the ATPase assay (Fig.10).
In the multiple motor gliding assay the K_m(ATP)-values for NcKIF1 434cys were 141.5 μM ATP and 222.8 μM ATP.

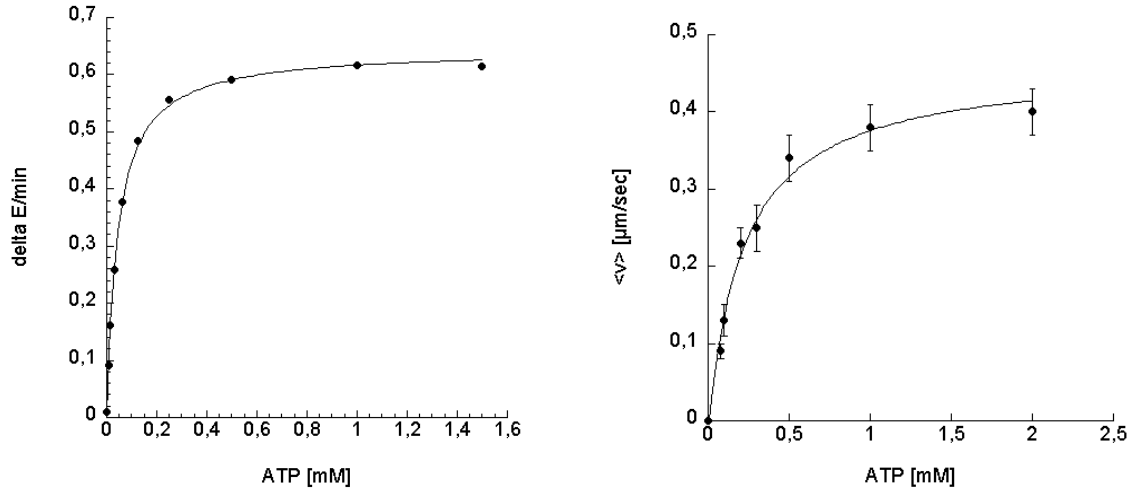


Fig.10. Determination of the K_m(ATP) for NcKIF1 434cys in the ATPase assay and the multiple motor gliding assay. The left graph shows the curve resulting from the measurements for the K_m(ATP) in the ATPase assay. The K_mATP was 42.8 μM ATP and 45.0 μM ATP for two independent protein preparations. The right graph shows the curve resulting from the measurements for the K_m(ATP) in the multiple motor gliding assay. The K_m-values were 141.5 μM ATP and 222.8 μM ATP, respectively. The same preparations were used as for the determination of the K_m(ATP) in the ATPase assay.

Dividing of the K_m(ATP)-values for the multiple motor gliding assay and the ATPase assay (K_m(ATP) multiple motor gliding assay [μM ATP] / K_m(ATP) ATPase assay [μM ATP]) results in a ratio of 3.31 (141.5 μM ATP / 42.8 μM ATP) and 4.95 (222.8 μM ATP / 45.0 μM ATP; Fig.10), respectively (Leibler and Huse, 1993).

3.3.4. Molecular weight determination

The members of the UNC-104/KIF1 family are believed to be mostly monomeric proteins. Therefore, it was an interesting question whether NcKIF1 is a monomeric protein as well. For this, the molecular weight was determined using gel filtration and sucrose density gradient centrifugation (2.6.8.). The short construct, NcKIF1 434, the short construct with the cys-tag, NcKIF1 434cys and the full-length protein, NcKIF1 647, were examined. Two different protein preparations were tested for each.

In the gel filtration experiment a Superdex 200 gel filtration column was used. A standard curve was drawn from the Stokes radii of the standard proteins (ferritin, 450 kDa, r_{Stokes}=5.9 nm; aldolase, 158 kDa, r_{Stokes}=4.5 nm; BSA, 68 kDa, r_{Stokes}=3.55 nm; carboanhydrase, 29 kDa, r_{Stokes}=2.4 nm; cytochrome C, 12.5 kDa, r_{Stokes}=1.64 nm) and their elution volumes (ml). The

standard curve was fitted to a linear function. From the standard curve the Stokes radii of the NcKIF1 proteins could then be determined (Fig.11).

The Stokes radius of NcKIF1 434 was 3.67 nm +/- 0.13 nm (n=2) and for NcKIF1 434cys 3.50 +/- 0.05 nm (n=2).

For NcKIF1 647 the Stokes radius could not be determined. The NcKIF1 647 protein preparations eluted in the dead volume of the gel filtration column. This was possibly due to aggregation of the protein. A high-speed (100000 rpm, 10 min, 4°C, rotor TLA 100.3) centrifugation step before loading onto the column to remove possible aggregates did not change the elution pattern. In each experiment, the proteins were also loaded through a filter (syringe filter, 0.45 μm) onto the gel filtration column. This was performed to prevent aggregates and other things to enter the column. Still, NcKIF1 647 behaved as a high molecular weight aggregate.

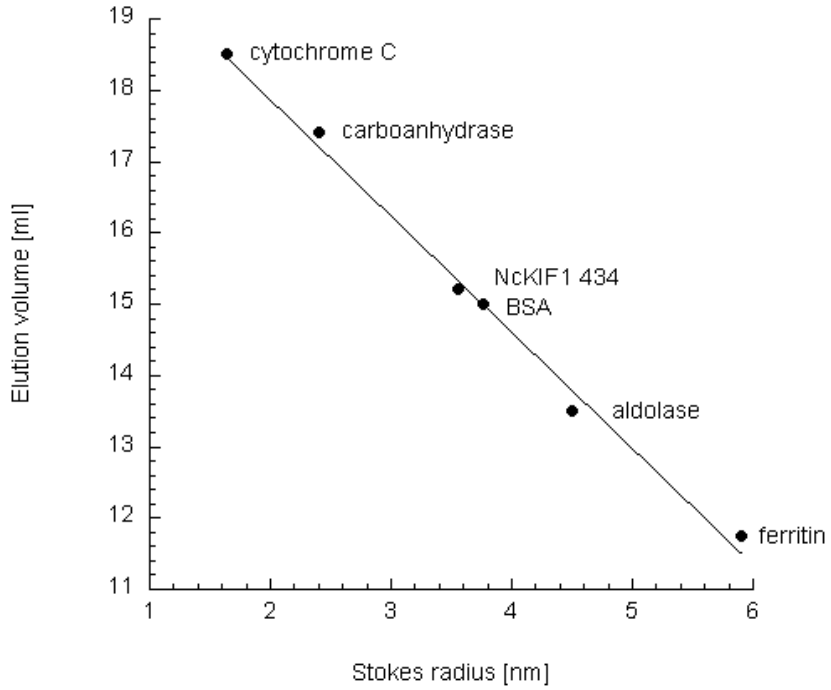


Fig.11. The gel filtration experiment. A standard curve from the Stokes radii of the standard proteins and their elution volume was drawn. From the elution volume of NcKIF1 and the standard curve the Stokes radius could be determined. NcKIF1 434 is shown here as an example. For NcKIF1 434cys about the same Stokes radius as for NcKIF1 434 was determined. The Stokes radius for NcKIF1 647 could not be determined because elution took place in the dead volume of the gel filtration column, indicating aggregation.

Sucrose density gradient centrifugation of NcKIF1 434, NcKIF1 434cys and NcKIF1 647 was performed (Fig.12 and Fig.13.) to determine the sedimentation coefficient.

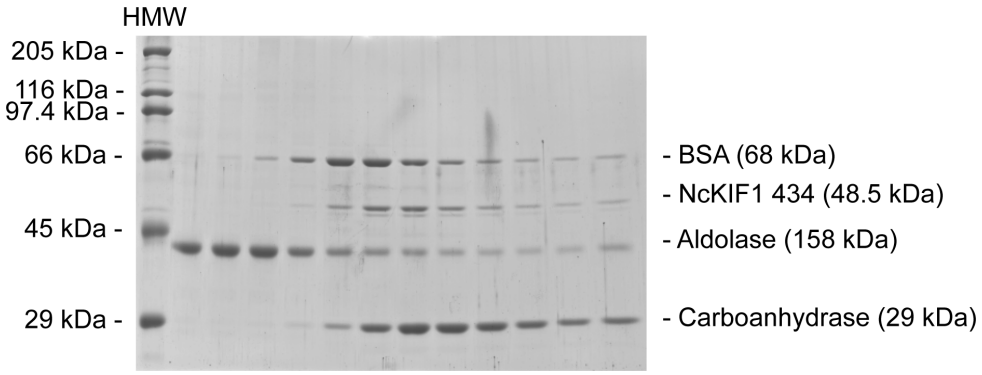


Fig.12. Sucrose density gradient centrifugation of NcKIF1 434. After centrifugation through a sucrose density gradient 300 μl fractions were collected from the bottom. The densest fraction (18%) is located on the left side of the PAA-gel and the lightest fraction (5%) on the right side. BSA, aldolase (a tetramer with a size of 158 kDa) and carboanhydrase were taken as standard proteins. There was no difference between NcKIF1 434 and NcKIF1 434cys.

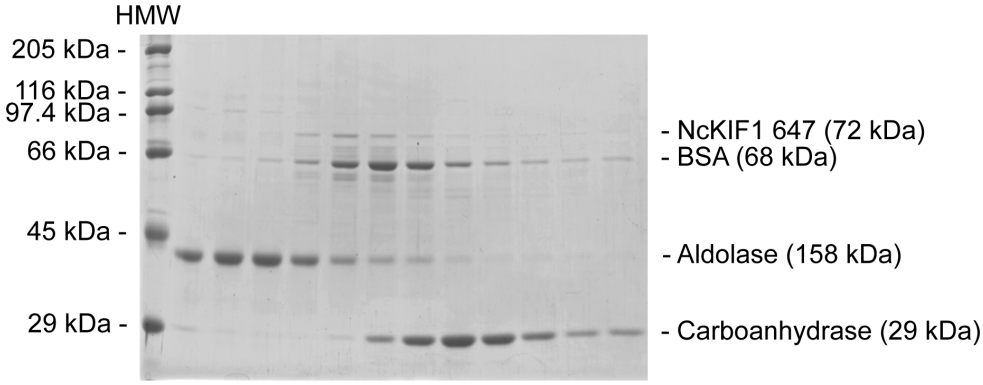


Fig.13. Sucrose density gradient centrifugation of NcKIF1 647. The locations of the fractions are the same as described in Fig.12 as well as the standard proteins. There was no pellet of NcKIF1 647 on the bottom of the tube after the centrifugation.

The protein bands in the PAA-gel were evaluated densitometrically with the NIH Image programme. The density of the protein bands as determined by the integrated pixel intensity were plotted against the 300 μl-fractions using the KaleidaGraph programme (Fig.14). From the maxima of the curves and the known Svedberg constants ($S_{w,20}$-value) of the standard proteins (aldolase, $S_{w,20}$=7.4; carboanhydrase, $S_{w,20}$=3.2 and BSA, $S_{w,20}$=4.22) a standard curve was drawn. The $S_{w,20}$-value of the kinesin was then calulated from the standard curve (Fig.15).

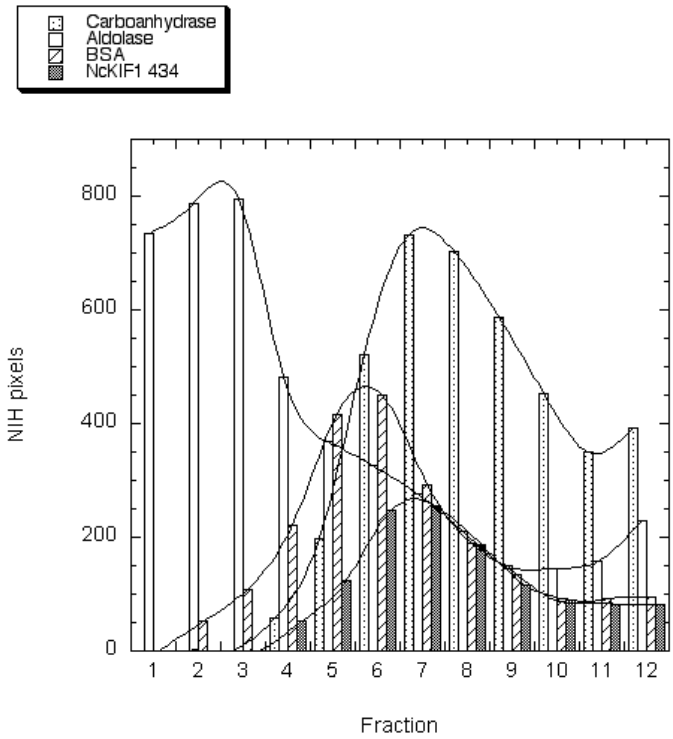


Fig.14. The NIH pixels (density of the protein bands) were plotted against the 300 μl-fractions. A standard curve was drawn from the maxima of the curves and the known Svedberg constants ($S_{w,20}$-values) of the standard proteins aldolase, carboanhydrase and BSA. This curve was fitted to a linear function (Fig.15).

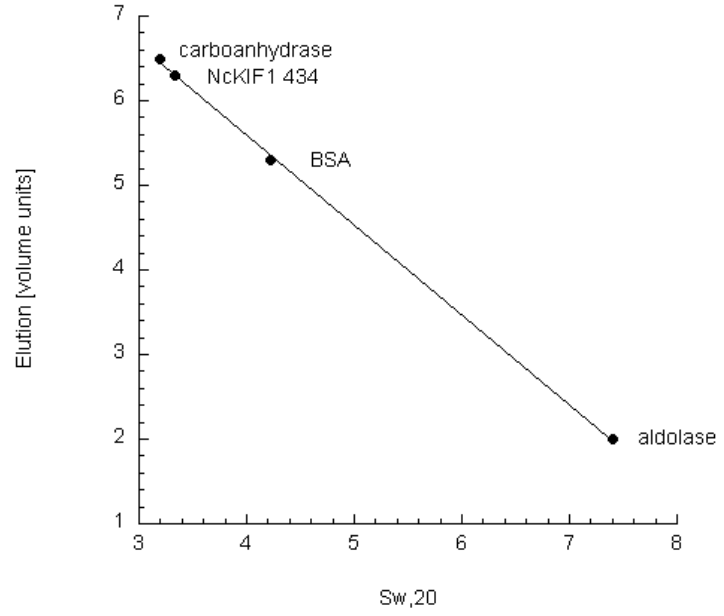


Fig.15. From the standard curve the $S_{w,20}$-value for the kinesin was calculated. The location of NcKIF1 434 within the standard curve is shown here as an example.

The $S_{w,20}$-value for NcKIF1 434 was 3.22 +/- 0.17 (n=2), for NcKIF1 434cys 3.22 +/- 0.06 (n=2) and for NcKIF1 647 4.89 +/- 0.32 (n=2).

The $S_{w,20}$-value for NcKIF1 434 was 3.22 +/- 0.17 (n=2), for NcKIF1 434cys 3.22 +/- 0.06 (n=2) and for NcKIF1 647 4.89 +/- 0.32 (n=2).

From the Stokes radius and the $S_{w,20}$-value the molecular weight of the protein can be calculated as described in 2.6.8.. For NcKIF1 434 a molecular weight of 48.8 +/- 4.3 kDa versus 48.5 kDa calculated by the GCG programme was determined and for NcKIF1 434cys 46.5 +/- 0.2 kDa versus 49.5 kDa. These results show the short motor domain construct to be a monomeric protein. For the full-length kinesin, NcKIF1 647, the native molecular weight could not be determined because aggregation took place in the gel filtration. For the determination of the molecular weight both experiments, gel filtration and density gradient centrifugation, are required. From the density gradient centrifugation of NcKIF1 647 alone, the oligomerisation state can not be determined. Therefore, the oligomerisation state of NcKIF1 should be tested again on constructs that are longer than NcKIF1 434, but do not show the degradation of NcKIF1 647.
To exclude that after sucrose density gradient centrifugation the full-length protein, NcKIF1 647, has pelleted and is then found on the bottom of the tube, the bottom was examined for a pellet after taking the fractions. No pellet could be observed on the bottom of the tube. Then, the bottom was rinsed thoroughly and carefully with 50 μl of 1 x Laemmli sample buffer to dissolve a putative protein pellet and 20 μl of this solution were loaded onto a PAA-gel. The analysis of the PAA-gel showed that NcKIF1 647 had not sedimented to the bottom. This excludes that NcKIF1 647 is on the bottom of the tube, whereas just a small fraction of the protein is in solution and can be found in the fractions. The aggregation in the gel filtration could be due to an interaction with the column material. The degradation of NcKIF1 647 could also be one reason.

3.4. In vivo studies of NcKIF1

3.4.1. Northern blot analysis

To test the expression of NcKIF1 in vivo, Northern blot analysis from 20 µg of *N. crassa* mycelial total RNA was performed. The probe was generated from the λ10 vector corresponding to the DNA sequence of NcKIF1 399. The sequence contains the motor domain of NcKIF1 and the following 14 amino acids (see also Fig.4). The probe was generated by random hexamer priming. As a control, the Northern blot was also performed with a probe to detect NcKin. For this purpose, the tail of NcKin was selected.

In the Northern blot a signal was seen for NcKIF1 at 2800 bp, in aggreement with the size of the cDNA. For NcKin a signal at 3500 bp was detected. This size corresponds to the expected size of the cDNA for NcKin. The size of the bands were estimated from the size of the ribosomal bands. Therefore, they are just estimations but correspond well to the expected sizes of the cDNA (Fig.16).
Northern blot analysis of *N. crassa* total RNA suggests that NcKIF1 is expressed in vivo and should be present in mycelia.

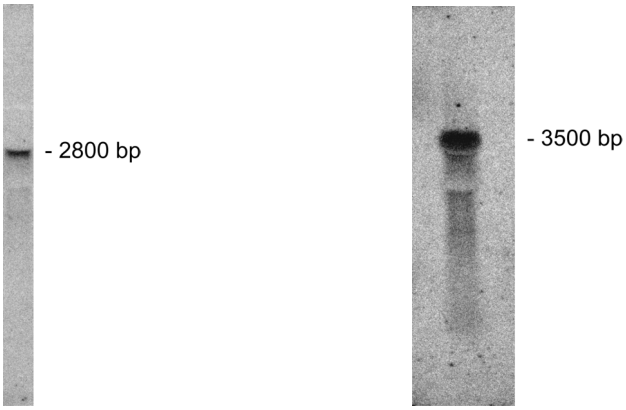


Fig.16. Left: Northern blot of NcKIF1. Analysis of 20 μg of mycelial total RNA showed a signal consistent with the size of the cDNA found in a screen of the mycelial cDNA library M-1. The motor domain of NcKIF1 was used as a probe. Right: Northern blot of NcKin. The tail of NcKin was used as a probe. The signal agrees with the expected size of the cDNA for NcKin. The size of the bands were estimated from the size of the ribosomal bands. The probes were generated using random hexamer priming.

3.4.2. Screening of different cDNA libraries

Four different cDNA libraries of *N. crassa* were screened to see if NcKIF1 can be detected in one of these libraries by PCR. Two mycelial cDNA libraries (M-1 and 74 A West) as well as a conidial (C-1) and a perithecial (P-1) cDNA library were screened. The libraries were obtained from the Fungal Genetics Stocks Center (FGSC, the University of Kansas Medical Center, USA). A PCR (30 cycles) was performed using 1 μl of the phage library in a 25 μl-reaction and the primers KIFSMe, NcKflHC for NcKIF1 and NKNde5, NK433- for NcKin. This is a very rough screen just to see if a signal can be obtained from one of the libraries. No signal could be obtained for NcKIF1 in one of these libraries, whereas NcKin could be detected in all of them. The cDNA of NcKIF1 was already found in the mycelial cDNA library M-1 (see 3.1.1.) but seems at least below the detection limit under these conditions. A signal from one of the libraries could have indicated a higher expression level, especially in case of the other developmental stages.

3.4.3. Detection of NcKIF1 in *N. crassa* crude extracts

To determine the presence of NcKIF1 in *N. crassa* crude extracts an antibody was raised against NcKIF1 399 containing the conserved motor domain and the following 14 amino acids of NcKIF1 (sequence see Fig.4). Prior to immunization the sera of 5 rabbits were tested against *E. coli* and *N. crassa* crude extracts by Western blotting to exclude the presence of antibodies against *E. coli* and *N. crassa* proteins and a high background. Two rabbits were selected for immunization and the sera tested from day 1, 70 and 100 of immunization against the antigen preparation (NcKIF1 399) by Western blotting. The serum giving the best signal was used for affinity purification. The sensitivity of the affinity-purified antibody was tested in a dot blot test. The detection limit of the affinity-purified antibody was < 230 pg.

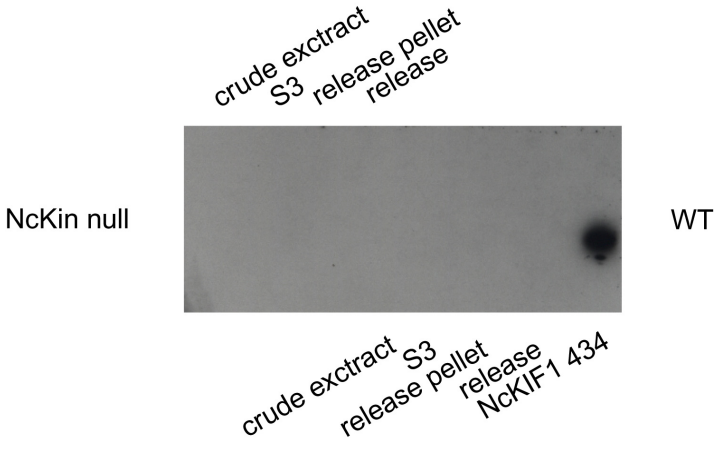
For the detection of NcKIF1 from *N. crassa* mycelia two different methods for the preparation of the crude extracts were used. With the first method, the crude extract corresponds more to a cytosolic extract. A shaking culture of minimal medium was inoculated with conidia and grown for 16 h at 25°C under constant light. After that time the mycelium was harvested and ground with sea sand, AP-100, DTT and protease inhibitors for 15 min at 4°C. Reducing the grinding time did not improve the results. Then centrifugation was carried out at 4°C for 20 min at 12000 rpm and following that, centrifugation of the supernatant at 4°C for 60 min at 45000 rpm. With this method, after the second centrifugation the layers of the supernatant were as follows: fatty top layer, middle layer, vacuole-rich layer above the bottom and the pellet. As crude extract the middle of the supernatant was used.

With the second method 4 l of minimal medium was inoculated with conidia and grown under permanent air supply for 16 h at 25°C and constant light. After harvesting the mycelium was ground as described in the first method for 5 min at 4°C. Then centrifugation took place twice for 10 min at 12000 rpm at 4°C and after that, further two centrifugation steps were carried out for 15 min at 4°C. After each centrifugation step the supernatant was taken for the next centrifugation. The supernatant after the last centrifugation was taken as crude extract. This crude extract corresponds more to a cell extract.

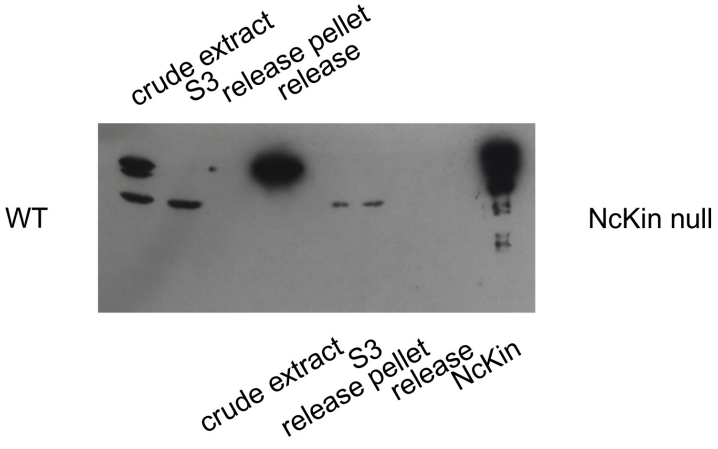
N. crassa wildtype and *N. crassa* NcKin null mutant were grown and used for the examinations. This was done to include the possibility of upregulation of NcKIF1 in the NcKin null mutant.

First, the crude extract prepared with the first method was examined by Western blotting with the affinity-purified NcKIF1 motor domain antibody (see above). *N. crassa* wildtype as well as NcKin null mutant were examined. NcKIF1 could not be detected. The quality of the crude extracts was controlled by detecting NcKin in the Western blot using an affinity-purified

NcKin antibody (α-105) and NcKin as control protein. It was then tried to enrich NcKIF1 from the crude extracts by binding to microtubules with AMP-PNP and release with an excess of ATP. This method was also used during the biotinylation procedure of the recombinant NcKIF1 protein (NcKIF1 434 and NcKIF1 647) and the Cy3-labelling (see 2.6.10. and 2.6.11.) and worked very well. After performing the Western blot no signal for NcKIF1 could be seen. Just the control protein, NcKIF1 434, could be detected with the motor domain antibody for NcKIF1 (Fig.17 A). Each step of the microtubule affinity enrichment method was examined (the crude extract; S3 - supernatant after microtubule-binding and centrifugation; release pellet and the release, which should contain the kinesins).



A



B

Fig.17. Western blot of *N. crassa* crude extract. Microtubule affinity enrichment was performed to detect NcKIF1. *N. crassa* wildtype and NcKin null mutant were used. In A an affinity-purified antibody raised against the motor domain of NcKIF1 and in B an affinity-purified antibody recognizing NcKin (α-105) were used. NcKIF1 434 and NcKin were used as control proteins. NcKIF1 could not be detected from *N. crassa* crude extracts even after microtubule affinity enrichment. WT – *N. crassa* wildtype; NcKin null – *N. crassa* NcKin null mutant.

A highly sensitive Western blotting substrate was used for the Western blot, and even exposing the blot on the film for 1 h did not change the results. As a control, NcKin was detected in the crude extracts and the following steps using the affinity-purified NcKin antibody α-105. The different steps of the microtubule affinity enrichment method can be followed very well (Fig.17 B). NcKin was used as control protein for this blot.

Western blotting was also performed on the supernatant after the first centrifugation step and the top fatty layer, the part above the bottom and the pellet. NcKIF1 could not be detected in either fraction.

NcKIF1 could also not be detected in crude extracts using the second method. The microtubule affinity enrichment did not work very well with the cell extract. The release showed no signal for NcKIF1 in the Western blot.

For the detection of NcKIF1, besides the antibody raised against the motor domain of NcKIF1 the samples were also examined using the MMR44-antibody and an affinity-purified antibody raised against NcKIF1 full-length protein purified from inclusion bodies of NcKIF1 (gift of Florian Fuchs and Benedikt Westermann). The MMR44-antibody is a peptide antibody against the conserved motif „VDLAGSE“ in the kinesin motor domain (Marks et al., 1994). Using these antibodies, no NcKIF1 was detected in both types of crude extracts as well as after microtubule affinity enrichment. Western blots were performed on at least two independently grown cultures of *N. crassa* mycelium and crude extracts prepared from them, as well as on preparations after microtubule affinity enrichment. In control Western blots with the α-105 antibody recognizing NcKin, a positive signal for NcKin was detected. With the MMR44-antibody no enrichment of a band in the size of NcKIF1 could be detected using the microtubule affinity enrichment. There was also no difference in the detection of NcKIF1 using *N. crassa* shaking cultures or 4 l-cultures.

To determine whether NcKIF1 is a membrane-bound protein, 1% Triton X-100 was added to the supernatant after the first centrifugation. The experiment was performed using the first method. A sample was taken from the supernatant after the first centrifugation, as well as from all layers after the second centrifugation including the pellet. No signal for NcKIF1 could be detected by Western blotting, indicating that NcKIF1 is probably not a membrane-bound protein.

In summary, NcKIF1 could not be detected in mycelial crude extracts of *N. crassa* wildtype as well as NcKin null mutant using Western blotting and microtubule affinity enrichment.

4. DISCUSSION

4.1. Structural observations

This study presents an initial characterization of NcKIF1, a new member of the UNC-104/KIF1 family of kinesin-like proteins. A sequence comparison to other known protein sequences revealed the closest homology of NcKIF1 to the members of the UNC-104/KIF1 family in the N-terminal part of the molecule, which turned out to be the motor domain. The amino acid sequence behind the motor domain however, did not show a similiarity to any known protein sequences. The NcKIF1 molecule has some interesting features. It has a shorter C-terminus than other members of the UNC-104/KIF1 family. It has a different K-loop in comparison to that of the mammalian representatives of this kinesin subfamily, which could point to a different motility mechanism of this molecule. With few exceptions, the members of the UNC-104/KIF1 family are known as monomeric kinesins. Thus, the UNC-104/KIF1 family takes up a special position amongst other members of the kinesin superfamily, which are usually composed of more than one polypeptide chain.
Comparison of the motor domain of NcKIF1 to the motor domains of other members of the UNC-104/KIF1 family of kinesin-like proteins shows a high similarity (63.5 % - 67.4 %, see Table 2; 3.1.2.). Structural elements typical of all kinesins, such as the P-loop (Saraste et al., 1990), switch I, switch II (Vale, 1996) or the microtubule binding site (Alonso et al., 1998; Woehlke et al., 1997) can be found in the motor domain of NcKIF1 (Sablin et al., 1996; Vale and Fletterick, 1997).
With 420 amino acids the motor domain of NcKIF1 is relatively large in comparison to the tail domain with 227 amino acids. It includes a 35 amino acid extension at the N-terminus which can not be found in other members of the UNC-104/KIF1 family. This corresponds to an approximate ratio of motor domain / tail domain of 1 : 0.5. The other known members of the UNC-104/KIF1 family contain a motor domain ranging from 354 to 368 amino acids in length. The lengths of the tail domains vary between 743 and 1843 amino acids, corresponding to a ratio of motor domain / tail domain of 1 : 2 to 1 : 5 (see Fig.4; 3.1.2.).
The members of the UNC-104/KIF1 family of kinesin-like proteins have an amino acid insertion in loop 12 of the motor domain, the so-called K-loop. This loop contains several positively charged amino acid residues (lysine or arginine) in sequence and is described to interact with the glutamate-rich, highly negatively charged C-terminal region of tubulin (E-hook) (Okada and Hirokawa, 2000). By molecular phylogenetic analysis it was shown that the

K-loop is conserved only in members of the UNC-104/KIF1 subfamily (known as mostly monomeric KIFs) and does not occur in dimeric (or tetrameric) KIFs. It was suggested that the K-loop plays a unique role in monomeric KIFs (Okada and Hirokawa, 1999).
The K-loop is surmised to work as an extra binding site for microtubules that dramatically increases the affinity to microtubules (Kikkawa et al., 2000). Furthermore, the interactions of the K-loop with the negatively charged E-hook of tubulin are thought to be essential for the processivity of the monomeric kinesins of the UNC-104/KIF1 subfamily (Okada and Hirokawa, 2000; Kikkawa et al., 2000).
Processive motion describes the walking of a kinesin molecule along a microtubule protofilament. Conventional kinesin is a highly processive dimeric motor which can take more than 100 steps along a microtubule before dissociating. Stepping is believed to occur by a ‚hand over hand' mechanism with one head always attached to the microtubule. In this model, the trailing head detaches and rebinds to the next open binding site on the microtubule, leading to an 8 nm movement for each step (Howard et al., 1989; Svoboda et al., 1993). This mechanism can not operate in the case of the monomeric kinesins of the UNC-104/KIF1 family. For the movement of these molecules along microtubules a different model was proposed. In this model, which was presented for mouse KIF1A, the monomeric kinesins of this family are suggested to move along microtubules by biased, one-dimensional diffusion. The interaction between the K-loop and the E-hook was suggested to play an essential role in anchoring of the motor to the microtubules, which would allow the diffusion of the monomeric motor along microtubules in the weak binding state (ADP in the active site) without diffusing away. This diffusional anchoring and the highly flexible structure of the E-hook was suggested to enable the small monomeric motor (motor domain of KIF1A ~6 nm) to surmount the interval between adjacent binding sites on the microtubule (8 nm) (Okada and Hirokawa, 2000; Okada and Hirokawa, 1999).
The sequence alignment of NcKIF1 with other members of the UNC-104/KIF1 family showed that NcKIF1 does not contain the same K-loop as other members (Fig.4, 3.1.2.). In the K-loop of NcKIF1 no clusters of lysine or arginine residues can be found as in the mammalian representatives of this family. This raises the question whether this K-loop can also interact with the negatively charged E-hook of tubulin as described in the model for mouse KIF1A (Okada and Hirokawa, 2000). It is possible that the mechanism that holds the NcKIF1 molecules on the microtubules during movement is different.
The sequence alignment of NcKIF1 with other known members of the UNC-104/KIF1 family showed also that behind the conserved motor domain of NcKIF1 the sequence of this

molecule differs from that of other members of this family. This was also one reason to isolate the NcKIF1 sequence again from a cDNA library of *N. crassa* to confirm this unusual tail sequence. The newly found sequence of NcKIF1 showed the same tail sequence as previously found in a cDNA library of *N. crassa* (Seiler, 1999). Furthermore, the part behind the conserved motor domain of NcKIF1 is not only different in sequence in comparison to other members of this family but also very short.

The tail domains are greatly divergent among kinesin superfamily proteins and reflect their different functions. The tail of kinesin is thought to bind cargo and accessory proteins and also to be involved in the regulation of the motor activity (Goldstein, 2001; Verhey et al., 2001). Cargo binding can also occur via adaptor proteins (Schnapp, 2003). The different tails of the kinesins are responsible for the cargo specificity. Taking this into account, it is possible that NcKIF1 transports a different cargo than other proteins of the UNC-104/KIF1 family. It is also possible that NcKIF1 is bound to an adaptor protein linking this molecule to its cargo. This would explain its short C-terminus. Further studies are necessary to determine the function of the short tail domain.

Recent analyses of fungal genomes revealed the presence of this type of „truncated“ UNC-104/KIF1-like proteins in other filamentous ascomycetes as well (Schoch et al., 2003). Short UNC-104/KIF1 kinesins may be specific for filamentous ascomycetes, because they could not be found in other organisms to date.

4.2. Biochemical studies

For basic biochemical studies, a short construct, NcKIF1 434, was generated containing the conserved motor domain and the following 14 amino acids. This protein could be bacterially expressed very well. The full-length protein, NcKIF1 647, was also bacterially expressed and included in the investigations.

To address the question whether NcKIF1 434 and the full-length protein, NcKIF1 647, are monomers, gel filtration and sucrose density gradient centrifugation were used. Most members of the UNC-104/KIF1 family of kinesin-like proteins are described to be monomeric proteins. Known exceptions are DdUnc104, which was shown to be a dimer, and HsKIF1C, which can also form dimers in vivo. Gel filtration and density gradient centrifugation showed NcKIF1 434 to be a monomeric protein. Gel filtration using the bacterially expressed full-length protein, NcKIF1 647, was not possible. The protein eluted in the dead volume of the gel filtration column, indicating aggregation. Gel filtration and sucrose density gradient

centrifugation performed with non-degraded longer constructs of NcKIF1 showed these constructs to be dimeric (personal communication, Sarah Adio and Günther Woehlke). This could be an explanation why the K-loop of NcKIF1 does not have the same appearance as other monomeric members of this family.

Microtubule-stimulated steady-state ATPase assays showed that NcKIF1 434 has a higher ATP turnover rate (k_{cat} = 34 +/- 6 sec^{-1}) than the full-length protein NcKIF1 647 (k_{cat} = 15 +/- 3 sec^{-1}).

From studies performed on shortened constructs of the conventional kinesin NcKin it is known that the length of the construct has an influence on the kinetics (Kallipolitou et al., 2001). Most of the shortened kinesin constructs showed higher ATP turnover rates in the microtubule-stimulated ATPase assay and decreased gliding velocities in the multiple motor gliding assay. The present data show that shortened constructs of NcKIF1 also can have higher ATP turnover rates (k_{cat}-values) in the ATPase assay compared to the full-length kinesin. Expression of the full-length protein NcKIF1 647 yielded a partially degraded protein. The k_{cat}-value for NcKIF1 647 shows therefore the sum of the activities of all the kinesin molecules present in the solution. Assuming that the truncated molecules in the protein preparation show a higher k_{cat}-value than the full-length molecule, the k_{cat}-value for NcKIF1 647 could be even lower than the measured value of 15 sec^{-1}.

Measurements of the basal ATPase activity of NcKIF1 434 and the microtubule-stimulated ATPase assay showed that the ATPase activity of NcKIF1 434 is activated 236-fold by microtubules. This confirms that NcKIF1 is a microtubule-activated ATPase.

NcKIF1 could also move microtubules in the multiple motor gliding assay. For NcKIF1 434 a gliding velocity of 0.46 μm/sec +/- 0.06 μm/sec was measured. NcKIF1 647 showed a higher gliding velocity with 0.60 +/- 0.05 μm/sec in this assay. Altering the salt concentration in the assay did not lead to a significant change in the gliding velocity of NcKIF1 434. The results from the multiple motor gliding assay indicate that the length of the molecule has an influence on the velocity.

In a theoretical analysis, Leibler and Huse compared the motors muscle myosin and kinesin and predicted the ratio of the K_m(ATP) for the multiple motor gliding assay and the K_m(ATP) for the ATPase assay to be approximately 10 or greater for myosin and 1-2 for kinesin (Leibler and Huse, 1993). They also divided molecular motors into porter and rower types with muscle myosins as rowers and kinesins as porters. The ratio indicates that in the case of porter type-motors 1-2 motor molecules are sufficient to move a microtuble (in the case of kinesins) in the multiple motor gliding assay. In motor molecules of the rower type however,

many motor molecules have to act together to generate movement, resulting in a ratio of 10 or greater for this type of motor. In vivo, it would mean that 1 (-2) motor molecules of the porter type, such as kinesins, can transport a cargo, for example, along a microtubule, whereas rower type-motor molecules, such as muscle myosin, work in large ensembles.
For NcKin in the multiple motor gliding assay K_m(ATP)- values of 187, 340 and 393 μM ATP and in the ATPase assay K_m(ATP)- values of 112, 145 and 194 μM ATP were measured (Steinberg and Schliwa, 1996), resulting in a ratio of ~2 for NcKin. NcKin was therefore classified as a molecular motor of the porter type. For NcKIF1 434 in the multiple motor gliding assay K_m(ATP)- values of 141.5 and 222.8 μM ATP and in the ATPase assay K_m(ATP)- values of 42.8 and 45.0 μM ATP were determined, resulting in a ratio of ~4. This could be explained by the fact that NcKIF1 434 is a monomeric protein (longer NcKIF1-constructs were shown to be dimeric) and therefore just contains one head. The ratio might indicate that several molecules of this kinesin construct are necessary to move a microtubule in the multiple motor gliding assay, whereas in the case of a dimeric kinesin just one kinesin molecule theoretically may be sufficient to translocate a microtubule. The ratio of ~4 for NcKIF1 434 is closer to the number given for the porter types of ~2 than for the rower types (≥ 10), thus suggesting that NcKIF1 is a motor of the porter type.

4.3. In vivo studies

In Northern blots with a probe directed against the motor domain of NcKIF1 a signal could be detected in concordance with the size of the cDNA of NcKIF1. The expression of NcKIF1 was weak in *N. crassa* mycelia. Presumably because of this weak expression, NcKIF1 could not be detected in *N. crassa* mycelial crude extracts by Western blotting. An enrichment of the kinesins present in the crude extracts of *N. crassa* was tried by attaching the kinesins to microtubules by AMP-PNP and subsequent release with ATP. This worked very well for the conventional kinesin NcKin. For NcKIF1, this enrichment did not lead to a detection of the native protein, even though this method worked very well for the recombinant full-length protein as well as for NcKIF1 434. Dissolving the membranous structures with 1% Triton X-100 did not lead to a detection of this protein, either.
Possible explanations for these findings are: First, since Northern blot analysis showed that the expression of NcKIF1 was weak, the amount of NcKIF1 protein was still below the detection limit of the Western blot (< 230 pg). Second, NcKIF1 is normally expressed at a low basal level. Only under certain conditions when the protein is needed, the expression

increases and leads to a higher amount of this protein in the cell. Third, the failure to detect NcKIF1 using the microtubule affinity enrichment is due to an interactor bound to the NcKIF1 molecule that inhibits the binding of the motor to microtubules.

The results suggest that NcKIF1 is expressed at a low basal level in mycelia of *N. crassa*, but it may be in an inactive state, which prevents binding to the microtubules. Furthermore, NcKIF1 could have a special function, for example in one of the developmental stages of *N. crassa*. This would also be supported by the unusual structure of this molecule with its small size, the large motor domain and the short divergent tail.

5. SUMMARY

The present work investigates a new member of the UNC-104/KIF1 family of kinesin-like proteins, NcKIF1, from the filamentous fungus *Neurospora crassa*. The cDNA of this molecule was isolated from a mycelial cDNA library of *N. crassa*, and codes for a protein with a predicted molecular weight of 72 kDa. To examine the basic properties of NcKIF1, a short construct encoding amino acids 1 – 434 and containing the conserved motor domain was cloned and expressed in *Escherichia coli*. The full-length protein, NcKIF1 647, was also cloned and bacterially expressed. The basic biochemical and biophysical behaviour of NcKIF1 was studied in vitro, and its expression in vivo was analysed in *N. crassa* mycelial crude extracts.

With a size of 72 kDa, NcKIF1 is exceptionally small in comparison to other members of the UNC-104/KIF1 family, which range from 122 kDa to 248 kDa per polypeptide chain. The motor domain of NcKIF1 is homologous to other representatives of this family. C-terminal of the conserved motor domain the homology ends, and the C-terminus of NcKIF1 shows no significant similarity to any previously known protein sequences. The motor domain of NcKIF1 is located at the N-terminus. This domain is larger than that of the other known members of this family, which is due in part to an N-terminal extension. Furthermore, the so-called K-loop is also present in NcKIF1, but does not contain several positively charged amino acid residues in sequence as, for example, in the mammalian representatives of this family. The C-terminal part of NcKIF1 with 227 amino acids is much shorter than that of other known members of the UNC-104/KIF1 family.

Bacterially expressed NcKIF1 is able to hydrolyze ATP as shown in the microtubule-stimulated ATPase assay. Measurements of the basal ATPase activity of NcKIF1 434 without addition of microtubules and the activity in the microtubule-stimulated ATPase assay showed that NcKIF1 434 is activated 236-fold by microtubules. NcKIF1 434 was able to move microtubules in the multiple motor gliding assay with a mean velocity of 0.46 μm/sec. The gliding velocity did not show a dependence on the salt concentration when different KCl concentrations were used in the assay. The Michaelis-Menten constant for ATP was 43.9 μM in the ATPase assay, and 182.2 μM in the gliding assay, indicating cooperativity. Size determination experiments showed NcKIF1 434 to be a monomeric protein. Most experiments were performed with NcKIF1 434, because the full-length protein, NcKIF1 647, contained degraded protein fragments.

Northern blot analysis showed that NcKIF1 is expressed in vivo in mycelia of *N. crassa*. Furthermore, the cDNA of NcKIF1 could be isolated from two different mycelial cDNA libraries of *N. crassa*, indicating that the gene is transcribed. However, the level of expression was very low.

5. ZUSAMMENFASSUNG

Die vorliegende Arbeit untersucht ein neues Mitglied der UNC-104/KIF1-Familie kinesin-ähnlicher Proteine, das NcKIF1 aus dem filamentösen Pilz *Neurospora crassa*. Die cDNA dieses Moleküls wurde aus einer myzelialen cDNA-Bibliothek von *N. crassa* isoliert und kodiert für ein Protein mit einem berechneten Molekulargewicht von 72 kDa. Um die Basiseigenschaften von NcKIF1 zu untersuchen wurde ein kurzes Konstrukt, welches die Aminosäuren 1 – 434 kodiert und die konservierte Motordomäne enthält, kloniert und in *Escherichia coli* exprimiert. Das Protein in der gesamten Länge, NcKIF1 647, wurde auch kloniert und bakteriell exprimiert. Es wurden in vitro Experimente durchgeführt, um das biochemische und biophysikalische Verhalten von NcKIF1 zu charakterisieren. Außerdem wurde untersucht, ob das Protein in vivo exprimiert wird und in myzelialen Rohextrakten von *N. crassa* nachgewiesen werden kann.

Mit einer Größe von 72 kDa ist NcKIF1 außergewöhnlich klein im Vergleich zu anderen Mitgliedern der UNC-104/KIF1-Familie (122 kDa bis 248 kDa pro Polypeptidkette). Die Motordomäne von NcKIF1 ist homolog zu anderen Vertretern dieser Familie. Die Homologie endet C-terminal von der konservierten Motordomäne und der C-Terminus von NcKIF1 zeigt keine signifikante Ähnlichkeit zu zuvor bekannten Proteinsequenzen. Die Motordomäne von NcKIF1 ist am N-Terminus lokalisiert. Diese Domäne ist größer als diejenige der anderen bekannten Mitglieder dieser Familie, was zum Teil auf eine N-terminale Erweiterung zurückzuführen ist. Weiterhin ist die sogenannte K-loop bei NcKIF1 auch vorhanden, jedoch enthält diese keine positiv geladenen Aminosäurenreste hintereinander wie z. B. bei den Säugetiervertretern dieser Familie. Der C-terminale Teil von NcKIF1 mit 227 Aminosäuren ist viel kürzer als bei anderen bekannten Mitgliedern der UNC-104/KIF1-Familie.

Bakteriell exprimiertes NcKIF1 ist in der Lage ATP zu hydrolysieren, wie aus dem Mikrotubuli-stimulierten ATPase Assay hervorgeht. Messungen der basalen ATPase Aktivität von NcKIF1 434 ohne Zugabe von Mikrotubuli und der Aktivität in dem Mikrotubuli-stimulierten ATPase Assay ergaben eine 236 fache Aktivierung von NcKIF1 434 durch Mikrotubuli. NcKIF1 434 konnte Mikrotubuli in dem „Multiplen Motor-Gleitassay“ mit einer mittleren Geschwindigkeit von 0.46 μm/sec bewegen. Eine Abhängigkeit der Gleitgeschwindigkeit von der Salzkonzentration konnte nicht beobachtet werden. Die Michaelis-Menten Konstanten für ATP waren 43.9 μM für den ATPase Assay und 182.2 μM für den Gleitassay, was auf Kooperativität hindeuten würde. Experimente zur Größenbestimmung zeigten, daß NcKIF1 434 als monomerisches Protein vorliegt. Die

meisten Experimente wurden mit NcKIF1 434 durchgeführt, da das vollständige Protein, NcKIF1 647, degradierte Proteinfragmente enthielt.
Northern Blot Analyse zeigte, daß NcKIF1 in vivo in Myzel von *N. crassa* exprimiert wird. Außerdem konnte die cDNA von NcKIF1 aus zwei verschiedenen myzelialen cDNA-Bibliotheken von *N. crassa* isoliert werden. Diese Ergebnisse zeigen, daß das Gen transkribiert wird, jedoch war die Expression sehr gering.

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APPENDIX:

Complete sequence alignment of NcKIF1 with other known members of the UNC-104/KIF1 family of kinesin-like proteins and NcKin, amino acids 1-332 (core motor domain). For description and references, see Results, Fig. 4..

```
1
  NcKIF1  MPNSLDVHQR QTRSNVSTPT LRPRDDTASS FVSKDPGANV RVVVRVRAFL
  UmKin3  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~MADSGNI KVVVRCRPMN
DdUnc104  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~MNV QVAVRVRPFN
Ceunc104  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~MSSV KVAVRVRPFN
 MmKIF1A  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~MAGASV KVAVRVRPFN
 MmKIF1B  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~MSGASV KVAVRVRPFN
MmKIF1Bb  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~MSGASV KVAVRVRPFN
 RnKIF1D  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~MAGASV KVAVRVRPFN
 HsKIF1C  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~MAGASV KVAVRVRPFN
  HsATSV  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~MAGASV KVAVRVRPFN
NcKin332  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~MSSSANSI KVVARFRPQN

          51
  NcKIF1  PRELERNAEC IVEMDPATER TSLLVPQETD FADARGARSR RVLEEKSFTF
  UmKin3  SRERNRGASN LIEFV..DQH QLILSP..PN EADTKENSKA TKKKSMPFSF
DdUnc104  SREKERNAEL IVQM..NNKS TILTRPSALR ...ANPLAAP TADDEKSFSF
Ceunc104  QREISNTSKC VLQVNGNT.. TTINGHSINK EN........ .....FSFNF
 MmKIF1A  SREMSRDSKC IIQMSGST.. TTIVNPKQPK ET........ ....PKSFSF
 MmKIF1B  SRETSKESKC IIQMQGNS.. TSIINPKNPK EA........ ....PKSFSF
MmKIF1Bb  SRETSKESKC IIQMQGNS.. TSIINPKNPK EA........ ....PKSFSF
 RnKIF1D  ARETSQDAKC VVSMQGNT.. TSIINPKQSR MF........ ....LKA.SF
 HsKIF1C  ARETSQDAKC VVSMQGNT.. TSIINPKQSK DA........ ....PKSFTF
  HsATSV  SREMSRDSKC IIQMSGST.. TTIVNPKQPK ET........ ....PKSFSF
NcKin332  RVEIESGGQP IVTFQGPD.. TCTVD...SK EA........ ....QGSFTF

          101
  NcKIF1  DKSFWSH.NT EDEHYATQEH VYDSLGEEFL DHNFEGYHTC IFAYGQTGSG
  UmKin3  DRAYDEHTEQ DD........ LFQYIGVELL QHAFNGFNTC VFAYGQTGSG
DdUnc104  DYSYWSYDS. NDPHFASQST VYNDLGKEVL KNAWDGFNCS IFAYGQTGSG
Ceunc104  DHSYWSF.AR NDPHFITQKQ VYEELGVEML EHAFEGYNVC IFAYGQTGSG
 MmKIF1A  DYSYWSHTSP EDINYASQKQ VYRDIGEEML QHAFEGYNVC IFAYGQTGAG
 MmKIF1B  DYSYWSHTSP EDPCFASQNR VYNDIGKEML LHAFEGYNVC IFAYGQTGAG
MmKIF1Bb  DYSYWSHTSP EDPCFASQNR VYNDIGKEML LHAFEGYNVC IFAYGQTGAG
 RnKIF1D  DYSYWSHTSV EDPQFASQQQ VYRDIGEEML LHAFEGYNVC IFAYGQTGAG
 HsKIF1C  DYSYWSHTST EDPQFASQQQ VYRDIGEEML LHAFEGYNVC IFAYGQTGAG
  HsATSV  DYSYWSHTSP EDINYASQKQ VYRDIGEEML QHAFEGYNVC IFAYGQTGAG
NcKin332  DRVFDMSCKQ SDIFDFSIKP TVDDI..... ...LNGYNGT VFAYGQTGAG
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          151
  NcKIF1  KSYTMMGT.. ..PDQPGLIP RTCEDLFQRI ASA.QDETPN ISYNVKVSYF
  UmKin3  KSHSMVGYA. ...QAKGIIP LTCARLFEDI NQK.TAADPN LKISVEVSYI
DdUnc104  KSYSMMGYG. ...EEKGIIP LICEELFQRI QSTPSNSNEQ TIYKTTVSYM
Ceunc104  KSYTMMG.KA NDPDEMGIIP RLCNDLFARI DNN.NDK..D VQYSVEVSYM
 MmKIF1A  KSYTMMG.K. QEKDQQGIIP QLCEDLFSRI NDT.TND..N MSYSVEVSYM
 MmKIF1B  KSYTMMG.K. QEESQAVIIP QLCEELFEKI NDN.CNE..E MSYSVEVSYM
MmKIF1Bb  KSYTMMG.K. QEESQAVIIP QLCEELFEKI NDN.CNE..E MSYSVEVSYM
 RnKIF1D  KSYTMMG.R. QEPGQQGIVP QLCEDLFSRV NVN.QSA..Q LSYSVEVSYM
 HsKIF1C  KSYTMMG.R. QEPGQQGIVP QLCEDLFSRV SEN.QSA..Q LSYSVEVSYM
  HsATSV  KSYTMMG.K. QEKDQQGIIP QLCEDLFSRI NDT.TND..N MSYSVEVSYM
NcKin332  KSYTMMGTSI DDPDGRGVIP RIVEQIFTSI LSS.A.A..N IEYTVRVSYM

          201
  NcKIF1  EVYNEHVRDL LAPVVPNKPP YYLKVRESPT EGPYVKDLTE VPVRGLEEII
  UmKin3  EIYNEKVRDL LNP....KNK GNLKVREHPS LGPYVEDLSK LVVASYPDIM
DdUnc104  EIYNEKVKDL LNP..NNNKT GGLKVRNNPS TGPYVEDLSK LAVKSFSEID
Ceunc104  EIYCERVKDL LNP....NSG GNLRVREHPL LGPYVDDLTK MAVCSYHDIC
 MmKIF1A  EIYCERVRDL LNP....KNK GNLRVREHPL LGPYVEDLSK LAVTSYNDIQ
 MmKIF1B  EIYCERVRDL LNP....KNK GNLRVREHPL LGPYVEDLSK LAVTSYTDIA
MmKIF1Bb  EIYCERVRDL LNP....KNK GNLRVREHPL LGPYVEDLSK LAVTSYTDIA
 RnKIF1D  EIYCERVRDL LNP....KSR GSLRVREHPI LGPYVQDLSK LAVTSYADIA
 HsKIF1C  EIYCERVRDL LNP....KSR GSLRVREHPI LGPYVQDLSK LAVTSYADIA
  HsATSV  EIYCERVRDL LNP....KNK GNLRVREHPL LGPYVEDLSK LAVTSYNDIQ
NcKin332  EIYMERIRDL LAP....QN. DNLPVHEEKN RGVYVKGLLE IYVSSVQEVY

          251
  NcKIF1  RWMRIGDGSR TVASTKMNDT SSRSHAVFTI MLKQIHHDLE TDDTTERSSR
  UmKin3  NLMDEGNKAR TVAATNMNET SSRSHAVFTL VLTQKRFDVQ TKLEAEKVSR
DdUnc104  MLMDEGSKAR TVASTNMNAT SSRSHAVFTI VFTQSKIDKT RGTAIDRVSK
Ceunc104  NLMDEGNKAR TVAATNMNST SSRSHAVFTI VLTQKRHCAD SNLDTEKHSK
 MmKIF1A  DLMDSGNKPR TVAATNMNET SSRSHAVFNI IFTQKRHDAE TNITTEKVSK
 MmKIF1B  DLMDAGNKAR TVAATNMNET SSRSHAVFTI VFTQKKQDPE TNLSTEKVSK
MmKIF1Bb  DLMDAGNKAR TVAATNMNET SSRSHAVFTI VFTQKKQDPE TNLSTEKVSK
 RnKIF1D  DLMDCGNKAR TVAATNMNET SSRSHAVFTI VFTQRSHDQL TGLDSEKVSK
 HsKIF1C  DLMDCGNKAR TVAATNMNET SSRSHAVFTI VFTQRCHDQL TGLDSEKVSK
  HsATSV  DLMDSGNKAR TVAATNMNET SSRSHAVFNI IFTQKRHDAE TNITTEKVSK
NcKin332  EVMRRGGNAR AVAATNMNQE SSRSHSIFVI TITQK..NVE TG..SAKSGQ

          301
  NcKIF1  IRLVDLAGSE RAKSTEATGQ RLREGSNINK SLTTLGRVIA ALADPKSSAS
  UmKin3  ISMVDLAGSE RANSTGATGA RLKEGANINR SLTTLGKVIA ALAIASSAVE
DdUnc104  ISLVDLAGSE RANSTGATGV RLKEGANINK SLSTLGKVIS ALAE......
Ceunc104  ISLVDLAGSE RANSTGAEGQ RLKEGANINK SLTTLGLVIS KLAE....ES
 MmKIF1A  ISLVDLAGSE RADSTGAKGT RLKEGANINK SLTTLGKVIS ALAEMDSGPN
 MmKIF1B  ISLVDLAGSE RADSTGAKGT RLKEGANINK SLTTLGKVIS ALAEV.....
MmKIF1Bb  ISLVDLAGSE RADSTGAKGT RLKEGANINK SLTTLGKVIS ALAEVDNCTS
 RnKIF1D  ISLVNLAGSE RADSSGARGM RLKEGANINK SLTTLGKVIS ALADL.....
 HsKIF1C  ISLVDLAGSE RADSSGARGM RLKEGANINK SLTTLGKVIS ALADM.....
  HsATSV  ISLVDLAGSE RADSTGAKGT RLKEGANINK SLTTLGKVIS ALAEMDSGPN
NcKin332  LFLVDLAGSE KVGKTGASGQ TLEEAKKINK SLSALGMVIN ALTD......
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          351
  NcKIF1  RPSSPVKSGR GRTPGPANSV VPYRDSVLTW LLKDSLGGNS KTAMIACISP
  UmKin3  PVKGAKK... .PKTASLDSF VPYRDSVLTW LLKDSLGGNS KTAMIAAISP
DdUnc104  .NSTSKKAV. .........F VPYRDSVLTY LLKETLGGNS KTIMIAAISP
Ceunc104  TKKKKSNK.. ........GV IPYRDSVLTW LLRENLGGNS KTAMLAALSP
 MmKIF1A  KNKKKKKT.. ........DF IPYRDSVLTW LLRENLGGNS RTAMVAALSP
 MmKIF1B  .SKKKKKT.. ........DF IPYRDSVLTW LLRENLGGNS RTAMVAALSP
MmKIF1Bb  KSKKKKKT.. ........DF IPYRDSVLTW LLRENLGGNS RTAMVAALSP
 RnKIF1D  .QSKKRKS.. ........DF IPYRDSVLTW LLKENLGGNS RTAMIAALSP
 HsKIF1C  .QSKKRKS.. ........DF IPYRDSVLTW LLKENLGGNS RTAMIAALSP
  HsATSV  KNKKKKKT.. ........DF IPYRDSVLTW LLRENLGGNS RTAMVAALSP
NcKin332  .....GKS.. ........SH VPYRDSKLTR ILQESLGGNS RTTLIINCSP

          401
  NcKIF1  TD..YDETLS TLRYADQAKR IRTRAVVNQV DGVS.AAERD AQIAAMAAEI
  UmKin3  AD..YEETLS TLRYADQAKK IKNKAVVNED PNAKLIRELK EELELLRTRV
DdUnc104  ADINYEESLS TLRYADSAKK IKTVAVVNED AQSKLIRELQ GEVERLRAMM
Ceunc104  ADINFDETLS TLRYADRAKQ IVCQAVVNED PNAKLIRELN EEVIKLRHIL
 MmKIF1A  ADINYDETLS TLRYADRAKQ IRCNAIINED PNNKLIRELK DEVTRLRDLL
 MmKIF1B  ADINYDETLS TLRYADRAKQ IKCNAVINED PNAKLVRELK EEVTRLKDLL
MmKIF1Bb  ADINYDETLS TLRYADRAKQ IKCNAVINED PNAKLVRELK EEVTRLKDLL
 RnKIF1D  ADINYEETLS TLRYADRTKQ IRCNAVINED PNARLIRELQ EEVARLRELL
 HsKIF1C  ADINYEETLS TLRYADRTKQ IRCNAIINED PNARLIRELQ EEVARLRELL
  HsATSV  ADINYDETLS TLRYADRAKQ IRCNAVINED PNNKLIRELK DEVTRLRDLL
NcKin332  SSYNDAETLS TLRFGMRAKS IKN~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~

          451
  NcKIF1  RQLQLVVSDS QTREKSALDA EQQLEEYQAR VRGLQQLMEE KSLVAEGKIR
  UmKin3  SGGG...... .........G ADGESNWDPS IPPDKQVVRY QTKTGE....
DdUnc104  DQGGQYHAND SKLMNSDYDE TVSTLN.... .......... ..........
Ceunc104  KDKGI.DVTD VQE.......  TPGKHKKGPK LPAH...... ..........
 MmKIF1A  YAQGLGDITD M......... .......... .......... .........T
 MmKIF1B  RAQGLG.... .......... .......... .........D IIDTSMGS..
MmKIF1Bb  RAQGLGDIID IDPLIDDYSG SGGKYLKDFQ NNKHRYLLAS ENQRPGNFST
 RnKIF1D  MAQGLS.... .......... .......... .....ASALG GLKVEEGSPG
 HsKIF1C  MAQGLS.... .......... .......... .....ASALE GLKTEEGSVR
  HsATSV  YAQGLGDITD M......... .......... .......... .........T
NcKin332  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~

          501
  NcKIF1  SLQTENEALR LHLKLALESL RNPIKVSSFP TTSLAMSAGD STVPLMAMGE
  UmKin3  .......... .......... .......... ...IKTVTKA ELQEQLEQSE
DdUnc104  .......... .......... .......... .......... ...EKIEQYE
Ceunc104  .......... .......... ........VH EQ........ ..LEKLQESE
 MmKIF1A  NALVGMSPSS SLSALSSRA. .AS..VSSLH ERILFAPGSE EAIERLKETE
 MmKIF1B  .....LTSSP SSCSLNSQVG LTS..VTSIQ ERIMSTPGGE EAIERLKESE
MmKIF1Bb  ASMGSLTSSP SSCSLNSQVG LTS..VTSIQ ERIMSTPGGE EAIERLKESE
 RnKIF1D  GVLPAASSPP APASPSSPPP HNGELEPSFS PSAEPQIGPE EAMERLQETE
 HsKIF1C  GALPAVSSPP APVSPSSPTT HNGELEPSFS PNTESQIGPE EAMERLQETE
  HsATSV  NALVGMSPSS SLSALSSRA. .AS..VSSLH ERILFAPGSE EAIERLKETE
NcKin332  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
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          551
  NcKIF1  VGTAH.GRKM ENKLVDDPFV DSGSGVTGDD DQGLICGHDD DYDTYEE.ED
  UmKin3  KIMSSLNESW EEKLTKTQEI QKEREK.ALE ELGISV.... DKGNVGVHTP
DdUnc104  KLMAELNKSW EEKLSEAEAI REDRMA.ALK DMGVAIK... ......VVS.
Ceunc104  KLMAEIGKTW EQKLIHTEEI RKQREEE.LR DMGLACAEDG T..TLGVFSP
 MmKIF1A  KIIAELNETW EEKLRRTEAI RMEREAL.LA EMGVAMREDG G..TLGVFSP
 MmKIF1B  KIIAELNETW EEKLRKTEAI RMEREAL.LA EMGVAIREDR G..DIGVFSP
MmKIF1Bb  KIIAELNETW EEKLRKTEAI RMEREAL.LA EMGVAIREDG G..TLGVFSP
 RnKIF1D  KIIAELNETW EEKLRKTEAL RMEREAL.LA EMG....SPG GWRTVGVFSP
 HsKIF1C  KIIAELNETW EEKLRKTEAL RMEREAL.LA EMGVAVREDG G..TVGVFSP
  HsATSV  KIIAELNETW EEKLRRTEAI RMEREAL.LA EMGVAMREDG G..TLGVFSP
NcKin332  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~

          601
  NcKIF1  DDDEDTIDLS EKAHDMNEYM SGLLKDLSMF RRKIGDDKTR FLDELGVRKP
  UmKin3  KKLPHLVNLN EDPL.MSECL IYQIK...PG HTLVGNLDSG PDVHIKLSGT
DdUnc104  .SIPHLINLN EDPL.MSESL IYYVKE...G KTRIGRSDSE IPQDIILNGL
Ceunc104  KKLPHLVNLN EDPL.MSECL IYYLKE...G VTSVGRPEAE HRPDILLSGE
 MmKIF1A  KKTPHLVNLN EDPL.MSECL LYYIKD...G VTRVGREDAE RRQDIVLSGH
 MmKIF1B  KKTPHLVNLN EDPL.MSECL LYYIKD...G ITRVGQADAE RRQDIVLSGA
MmKIF1Bb  KKTPHLVNLN EDPL.MSECL LYYIKD...G ITRVGQADAE RRQDIVLSGA
 RnKIF1D  KKTPHLVNLN EDPL.MSECL LYHIKD...G VTRVGQVDV. ...DIKLTGQ
 HsKIF1C  KKTPHLVNLN EDPL.MSECL LYHIKD...G VTRVGQVDM. ...DIKLTGQ
  HsATSV  KKTPHLVNLN EDPL.MSECL LYYIKD...G ITRVGREDGE RRQDIVLSGH
NcKin332  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~

          651
  NcKIF1  LGVRTNIM** ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
  UmKin3  KILNKHCMF. .......... .......... DHQDGLVTVT A.........
DdUnc104  NIHKEHCIFE NINGKVIISP SNNFMNNNNN KENSSSTTPT SSKSPSKPKS
Ceunc104  AILELHCEF. .......... .......INE D...GNVTLT M.........
 MmKIF1A  FIKEEHCIF. .......... .......RSD SRGGGEAVVT L.........
 MmKIF1B  HIKEEHCLF. .......... .......RSE RSNTGEVIVT L.........
MmKIF1Bb  HIKEEHCLF. .......... .......RSE RSNTGEVIVT L.........
 RnKIF1D  FIREQHCLF. .......... .......RSI PQPDGEVMVT L.........
 HsKIF1C  FIREQHCLF. .......... .......RSI PQPDGEVVVT L.........
  HsATSV  FIKEEHCVF. .......... .......RSD SRGGSEAVVT L.........
NcKin332  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~

          701
  NcKIF1  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
  UmKin3  .......... ....MP..DS MTMVNGKRLA PDEPKRLRSG YRVILGDFHV
DdUnc104  EKEKENNNDD DDGEKKLDRS YIYVNG..VE INKPTILTTG NRVILGNNHI
Ceunc104  .......... ....KP..NA SCYINGKQVT ..TPTVLHTG SRVILGEHHV
 MmKIF1A  .......... ....EPCEGA DTYVNGKKVT ..EPSILRSG NRIIMGKSHV
 MmKIF1B  .......... ....EPCERS ETYVNGKRVA ..HPVQLRSG NRIIMGKNHV
MmKIF1Bb  .......... ....EPCERS ETYVNGKRVA ..HPVQLRSG NRIIMGKNHV
 RnKIF1D  .......... ....EPCEGA ETYVNGKLVT ..EPLVLKSG NRIVMGKNHV
 HsKIF1C  .......... ....EPCEGA ETYVNGKLVT ..EPLVLKSG NRIVMGKNHV
  HsATSV  .......... ....EPCEGA DTYVNGKKVT ..EPSILRSG NRIIMGKSHV
NcKin332  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
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          751
  NcKIF1  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
  UmKin3  FRFNHPEEV. RKARDRVRST ......LALS TGE......A HNETLIDGDL
DdUnc104  FRFNNPEEAI KIARERNQTT GGIVSSTKSP VDQIMDYDFA LNELASIQG.
Ceunc104  FRYNDPQEA. RQSRHN..L. .......AAI AEQPIDWKYA QQELLDKQGI
 MmKIF1A  FRFNHPEQA. RQERER..TP ......CAET PAEPVDWAFA QRELLEKQGI
 MmKIF1B  FRFNHPEQA. RAEREK..TP ......SAET PSEPVDWTFA QRELLEKQGI
MmKIF1Bb  FRFNHPEQA. RAEREK..TP ......SAET PSEPVDWTFA QRELLEKQGI
 RnKIF1D  FRFNHPEQA. RLERERGVPP ......PPGP PSEPVDWNFA QKDWLEQQGI
 HsKIF1C  FRFNHPEQA. RLERERGVPP ......PPGP PSEPVDWNFA QKELLEQQGI
  HsATSV  FRFTHPEQA. RQERER..TP ......CAET PAEPVDWAFA QRELLEKQGI
NcKin332  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~

          801
  NcKIF1  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
  UmKin3  PSTRPDSPAS GDVDWTYARR EYTMAKLNGQ NVNFDNLNEE DLEKLFEDIS
DdUnc104  ..TLAMSKHI NDKQ.EYKKQ MRALYDQIRL QLENDCDPEV KEQREKLALL
Ceunc104  DLKADMEKKM LEMESQY.RR EKVELEQKMY HQTR..EYES MIENLQKQVD
 MmKIF1A  DMKQEMEQRL QELEDQY.RR EREEATYLLE QQRL..DYES KLEALQKQMD
 MmKIF1B  DMKQEMEKRL QEMEILY.KK EKEEADLLLE QQRLDADSDS GDDSDKRSCE
MmKIF1Bb  DMKQEMEKRL QEMEILY.KK EKEEADLLLE QQRL..DYES KLQALQRQVE
 RnKIF1D  DIKLEMEKRL QDLENQY.RK EKEEADLLLE QQRLYADSDS GEDSDKRSCE
 HsKIF1C  DIKLEMEKRL QDLENQY.RK EKEEADLLLE QQRLYADSDS GDDSDKRSCE
  HsATSV  DMKQEMEQRL QELEDQY.RR EREEATYLLE QQRL..DYES KLEALQKQMD
NcKin332  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~

          851
  NcKIF1  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
  UmKin3  RARSKKSMGS VLGRPESRAS LFDDNASESA SSVIRPYS.H GALTDDTSID
DdUnc104  AFRRWRSKVH RSKLLNKISF IILSLNEANA ISSTLNKKIN LSLKLYSVFP
Ceunc104  LAQSYISGGG SI...WEGER MLTSSLLEFP EELKWTSD.Q KRVVLKAAIK
 MmKIF1A  .SRYY....P EV...NEEEE .......EPE DEVQWTER.E CELALWAFRK
 MmKIF1B  ESWKLITSLR EKLPPSKLQT IVKKCGLPSS GKKREPIK.M YQIPQRRRL.
MmKIF1Bb  .TRSL....A AE...TTEEE .......EEE EEVPWTQH.E FELAQWAFRK
 RnKIF1D  ESWRLISSLR DELPPNTVQT IVKRCGLPSS GKRRAPRR.V YQIPQRRRLQ
 HsKIF1C  ESWRLISSLR EQLPPTTVQT IVKRCGLPSS GKRRAPRR.V YQIPQRRRLQ
  HsATSV  .SRYY....P EV...NEEEE .......EPE DEVQWTER.E CELALWAFRK
NcKin332  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~

          901
  NcKIF1  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
  UmKin3  PWSQAGSEMG SMRFSAGTPI .......... .......... ..........
DdUnc104  EPDQISDNIE PEIDWRKTQI LIKATDSSTG ESTLVTDQDF VDRIYLMREL
Ceunc104  WRYHQFTSVR DDLWG..NAI FVKEANAISV ELKKKVQFQF A...LLTDTM
 MmKIF1A  WKWYQFTSLR DLLWG..NAI FLKEANAISV ELKKKVQFQF V...LLTDTL
 MmKIF1B  SKDSKWVTIS DLKIQAVKEI CYEVALNDFR HSRQEIEALA I...VKMKEL
MmKIF1Bb  WKSHQFTSLR DLLWG..NAV YLKEANAISV ELKKKVQFQF V...LLTDTL
 RnKIF1D  GKDPRWATMA DLKMQAVKEI CYEVALADFR HGRAEIEALA A...LKMREL
 HsKIF1C  GKDPRWATMA DLKMQAVKEI CYEVALADFR HGRAEIEALA A...LKMREL
  HsATSV  WKWYQFTSLR DLLWG..NAI FLKEANAISV ELKKKVQFQF V...LLTDTL
NcKin332  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
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          951
  NcKIF1  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
  UmKin3  .......... .......... .KENAYTGAG ASSPALVAAS HRETESLRAK
DdUnc104  YQNDGRLDTE LPEDPFQFTF TKDSLIGVSH VYLKNTLYLV ESNRPVPILD
Ceunc104  YSPL.PPDL. LPP..GEDLT LRPYPKTVVA IQ........ .VQDLKNGA.
 MmKIF1A  YSPL.PPDL. LPPEAAKDRE TRPFPRTIVA VE........ .VQDQKNGA.
 MmKIF1B  CAMYGKKDP. NERDSWR.AV ARDVWDTVGV ...GDEKIED MMVTGKGGTD
MmKIF1Bb  YSPV.PPEL. LPSEMEKTHE DRPFPRTVVA VE........ .VQDLKNGA.
 RnKIF1D  CRTYGKPE.. GPGDAWR.AV ARDVWDTVGE EEGCG.GGGG GGEEGARGAE
 HsKIF1C  CRTYGKPD.. GPGDAWR.AV ARDVWDTVGE EEGGGAGSGG GSEEGARGAE
  HsATSV  YSPL.PPDL. LPPEAAKDRE KRPFPRTIVA VE........ .VQDQKNGA.
NcKin332  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~

          1001
  NcKIF1  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
  UmKin3  VREYEEKLTR MANGSPRLAD EPIEYSDTQK ALLRKVLVKW KAHTKVSMAE
DdUnc104  ENGNQKGYLN LLVSSSSTDI TESERGLYLE NPSNNKSLLL GKNLEITIGF
Ceunc104  .....THYWS IEKLKQRLEA MRDMYETDAE MSPADGDPMM DALMGTDPFY
 MmKIF1A  .....THYWT LEKLRQRLDL MREMYDRAAE VPSSVVEDCD NVVTGGDPFY
 MmKIF1B  VDDLKVHIDK LEDILQEVKK QNNMKDEEIK VLRNKMLKME KVLPLIGSQE
MmKIF1Bb  .....THYWS LDKLKQRLDL MREMYDRAGE VASSAQDDSE TTMTGSDPFY
 RnKIF1D  VEDLRAHIDK LTGILQEVKL QNSSKDRELQ ALRDRMLRME RVIPLTQDLE
 HsKIF1C  VEDLRAHIDK LTGILQEVKL QNSSKDRELQ ALRDRMLRME RVIPLAQDHE
  HsATSV  .....THYWT LEKLRQRLDL MREMYDRAAE VPSSVIEDCD NVVTGGDPFY
NcKin332  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~

          1051
  NcKIF1  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
  UmKin3  DALCKAVLVK EANVISKELA KRVTYQYTIV DDFPLAVPTS GVEAIAGLTE
DdUnc104  EGFSEFIDEN KFSDVFIKFN FPNQNGTIVD TFLTEPQPIS AFIDQKRIVI
Ceunc104  DRF....... .......... .......... .......... ..........
 MmKIF1A  DRF....... .......... .......... .......... ..........
 MmKIF1B  QKSQGSHKTK EPLVAGANSV .....SDNGV S.KGESGELG KEERVSQLMN
MmKIF1Bb  DRFHWFKLVG SSPIFHGCVN ERLADRTPSP TFSTADSDIT ELADEQQDAM
 RnKIF1D  DDNEESGLVT WAPPEGSEAV EEAVSNDHSP AVRPSSPPQS SWERVSRLME
 HsKIF1C  DENEEGGEVP WAPPEGSEAA EEAAPSDRMP SARPPSPPLS SWERVSRLME
  HsATSV  DRF....... .......... .......... .......... ..........
NcKin332  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~

          1101
  NcKIF1  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
  UmKin3  FDDVSDPDLA SCAKPCM... .....GIKVL DYLHSTCYVW SMPKFEQRLQ
DdUnc104  TSLTESLINL LQTQYVSFEI RGHKKS.KQQ PKLTSSSSSA STTSSSSKNQ
Ceunc104  .......... .......... .......... ......PWFR MVGRAFVYLN
 MmKIF1A  .......... .......... .......... ......PWFR LVGRAFVYLS
 MmKIF1B  GDPAFRRGRL RWMRQEQIRF KNLQ.QQEIT KQLRRQN... .VPHRFIPPE
MmKIF1Bb  EDFDDEAFVD DTGSDAGTEE GSELFSDGHD PFYDRSPWFI LVGRAFVYLS
 RnKIF1D  EDPAFRRGRL RWLKQEQLRL QGLQGSGGRG GGLRRP.... ..PARFVPPH
 HsKIF1C  EDPAFRRGRL RWLKQEQLRL QGLQGSGGRG GGLRRP.... ..PARFVPPH
  HsATSV  .......... .......... .......... ......PWFR LVGRAFVYLS
NcKin332  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
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          1151
  NcKIF1  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
  UmKin3  KMRNLYTFVD KPEYSKHLNW SDPFYEAPHP TYAFVASTLV PLTPLSRQLS
DdUnc104  PMLENFEFLA TLNILESEKN TGTDDQYKPV HILEDPDVYN THLPSVTFRL
Ceunc104  NLLHNVPLIH KVAVVNEKGE VKGYLKVAIE PVQKD.EVIN QKKGVRQ..T
 MmKIF1A  NLLYPVPLVH RVAIVSEKGE VKGFLRVAVQ AISADEEAPD YGSGVRQSGT
 MmKIF1B  NRKPRFPFKS NPKHRNSWSP G.......TH IIITEDEVIE LRIP..KDEE
MmKIF1Bb  NLLYPVPLIH RVAIVSEKGE VRGFLRVAVQ AIAADEEAPD YGSGIRQSGT
 RnKIF1D  DCKLRFPFKS NPQHRESW.P GMGSGEAPGP .QPPEEVTAP PPPPNRRPPS
 HsKIF1C  DCKLRFPFKS NPQHRESW.P GMGSGEAPTP LQPPEEVTPH PATPARRPPS
  HsATSV  NLLYPVPLVH RVAIVSEKGE VKGFLRVAVQ AISADEEAPD YGSGVRQSGT
NcKin332  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~

          1201
  NcKIF1  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
  UmKin3  AK..YRLPLH DRHTAKQIGW CSVSVKFVSL SPVPVSARAG GTALPAPSGS
DdUnc104  KKDKTNRQIL FKVIKNESNS IIKECKSARI SDIKI..... FGKRDNPLLS
Ceunc104  AKLHFRKEDF LKSHKN.... .......... .........G ETSD......
 MmKIF1A  AKISFDDQHF EKFQSESCPV VGMSRSGTSQ EELRIVEGQG QGADAGPSAD
 MmKIF1B  GRKENKEESQ EKVGRAASRD VQSAWGTRSQ DHIQVSKQHI SNQQPPPQLR
MmKIF1Bb  AKISFDNEYF NQSDFSS... AAMTRSGLSL EELRIVEGQG QSSEVISPPE
 RnKIF1D  PRRPHRPRRN SLDGGSRSRG GGST..QPEP QHLRPQKHNS YPQQPQPYPA
 HsKIF1C  PRRSHHPRRN SLDGGGRSRG AGSA..QPEP QHFQPKKHNS YPQPPQPYPA
  HsATSV  AKISFDDQHF EKFQSESCPV VGMSRSGTSQ EELRIVEGQG QGADVGPSAD
NcKin332  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~

          1251
  NcKIF1  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
  UmKin3  RNPSSPTSSS CTNGIV...N PLVGQKLGFQ ILVDAISGIS SDDFASIHLQ
DdUnc104  SSATPNTPNT PNNSRIAGIQ NTPGTPMTPY SNQTNNQQSS SSQPPLPQQQ
Ceunc104  .......... .........S DRLAFP.... ...EHMQEEV EFCFRVVVLQ
 MmKIF1A  EVNNNTCSAV PPEG.LMDSP EKAALDGPLD TALDHLRLGS TFTFRVTVLQ
 MmKIF1B  WRSNSLNNGQ PKTT..RCQA TASSESLNSH SGHPTADLQT FQAKRHIHQH
MmKIF1Bb  EVNRMNDLDL .KSGTLLDG. .KMVMEGFSE EIGNHLKLGS AFTFRVTVLQ
 RnKIF1D  QR.PG.PRYP PYTTPPRMRR QRSAPDL.KE SGAAV~~~~~ ~~~~~~~~~~
 HsKIF1C  QRPPG.PRYP PYTTPPRMRR QRSAPDL.KE SGAAV~~~~~ ~~~~~~~~~~
  HsATSV  EVNNNTCSAV PPEGLLLDSS EKAALDGPLD AALDHLRLGN TFTFRVTVLQ
NcKin332  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~

          1301
  NcKIF1  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
  UmKin3  VKLSSFAGNE LGKDEIYTSI PVDLVNQESL AEVRLRRTLS FV....LTPE
DdUnc104  GTPYNPQSNN .......... PNVISNAPPT PNSNLLKDLS LAANVQTSSS
Ceunc104  AIDVADTYSD VFCQFNFLHR HDEAFSTEPM KNS..KSPLT FEHTQNLHIK
 MmKIF1A  ASSISAEYAD IFCQFNFIHR HDEAFSTEPL KNTGRGPPLG FYHVQNIAVE
 MmKIF1B  RQPYCNYNTG GQVEGSTASC CQKQTDKPSH CNQFVTPPRM RRQFSAPNLK
MmKIF1Bb  ASGILPEYAD IFCQFNFLHR HDEAFSTEPL KNNGRGSPLG FYHVQNIAVE
 RnKIF1D  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
 HsKIF1C  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
  HsATSV  ASSISAEYAD IFCQFNFIHR HDEAFSTEPL KNTGRGPPLG FYHVQNIAVE
NcKin332  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
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          1351
  NcKIF1  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
  UmKin3  TIQWLRTGAA PIEVYAKLRP HYLVALEQHD SARESEGQQH AAAFVPLHDD
Ceunc104  MSKTFLHYLH HFPIIFEVFG HFQPKSEQFN F..ERQNSAL GRRLSTKLTF
DdUnc104  SSSSSLNVLL NNQQQQQQSQ QSQQQQQQQS QQSSETSSTT NSITNSASNS
 MmKIF1A  VTKSFIEYIK SQPIVFEVFG HYQ....QHP F..PPLCKDV LSPLRPSRRH
 MmKIF1B  AGRETTV~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
MmKIF1Bb  VTESFVDYIK TKPIVFEVFG HYQ....QHP L..HLQGQDL NSPPQPSRRF
 RnKIF1D  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
 HsKIF1C  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
  HsATSV  VTKSFIEYIK SQPIVFEVFG HYQ....QHP F..PPLCKDV LSPLRPSRRH
NcKin332  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~

          1401
  NcKIF1  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
  UmKin3  SRSLTAGQQK TMSNTIKERL SENEMRNEER HFILASVQIC ELDASGEYMP
DdUnc104  SLSLLVNNQT TNSNNSGGNI FEIPVLSCTD DSVLLLWKTN ..DPSFIFNQ
Ceunc104  QQPSLVISTP VKSKKANAPI QNNNASVKSK HDLLVWFEIC ELANNGEYVP
 MmKIF1A  FPRVMPLSKP VPATKLSTMT RPSPGPCHCK YDLLVYFEIC ELEANGDYIP
 MmKIF1B  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
MmKIF1Bb  FPPPMPLSKP VPATKLNTMN KTTLGQSMSK YDLLVWFEIS ELEPTGEYIP
 RnKIF1D  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
 HsKIF1C  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
  HsATSV  FPRVMPLSKP VPATKLSTLT RPCPGPCHCK YDLLVYFEIC ELEANGDYIP
NcKin332  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~

          1451
  NcKIF1  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
  UmKin3  VPVRASSALD .PGSFFLRQG LQRKLVLQL. AHDSGRQFLW SRVTKLELAD
DdUnc104  KTRKGDKILF KLTFDLLIQG FPDVVSISKD IAIKILSSES MPSATMPDGT
Ceunc104  TIVDHAQGLP THGIFLLHQG IQRRIKITI. CHEKG.ELKW KDCQELVVGR
 MmKIF1A  AVVDHRGA.. CMGTFLLHQG IQRRITVTL. LHETGSHIRW KEVRELVVGR
 MmKIF1B  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
MmKIF1Bb  AVVDHTAGLP CQGTFLLHQG IQRRITVTI. IHEKGSELHW KDVRELVVGR
 RnKIF1D  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
 HsKIF1C  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
  HsATSV  AVVDHRGGMP CMGTFLLHQG IQRRITVTL. LHETGSHIRW KEVRELVVGR
NcKin332  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~

          1501
  NcKIF1  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
  UmKin3  VRLLDSRGRV HGGKASDAVQ LKT.....PL KQQSVEFANN GTSQLELWAW
DdUnc104  SSSSMSNLLD KFKTHFKGES ILSEPSIHAG SVFSINLTKS RQQEHQNRIG
Ceunc104  IRAGPEW.AG GDDVDVLSLG LFPGTFMEFS MDD....... .RTFFQFEAA
 MmKIF1A  IRNTPETDEA LIDPNILSLN ILSSGYVHPA QDDRVFFGND TRTFYQFEAA
 MmKIF1B  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
MmKIF1Bb  IRNKPEVDEA AVDA.VLSLN IISAKSLKAA H.......SS SRTFYRFEAV
 RnKIF1D  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
 HsKIF1C  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
  HsATSV  IRNTPETDES LIDPNILSLN ILSAGYIHPA HDD....... .RTFYQFEAA
NcKin332  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
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          1551
  NcKIF1  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
  UmKin3  WDSSVHDSLH LN.RTTSAGH RVLIRLSFEI QVDRCSAPAA FSMDLAVSIN
DdUnc104  EMIDAHQENI LKLGYAMKME KLRQELDLRE KLTNLKEKTI DSTNTDDVNA
Ceunc104  WDSSLHNSPL LN.RVSNYGD QIYMTLSAYM ELDGCAQPAV VTKDLCLLIY
 MmKIF1A  WDSSMHNSLL LN.RVTPYRE KIYMTLSAYI EMENCTQPAV ITKDFCMVFY
 MmKIF1B  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
MmKIF1Bb  WDSSLHNSLL LN.RVTPYGE KIYMTLSAYL ELDHCIQPAV ITKDVCMVFY
 RnKIF1D  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
 HsKIF1C  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
  HsATSV  WNSSMHNSLL LN.RITPYRE KIYMTLSAYI EMENCTQPAV VTKDFCMVFY
NcKin332  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~

          1601
  NcKIF1  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
  UmKin3  GRDAKPPGRL MSF....... IEGSTSMTKT SAIFEVRLVP PMMKRPCELW
DdUnc104  ANGVAESSNS STIDVEEIVK KMLLMNSTHQ QQQQNFSSPS STSPTLVNGE
Ceunc104  ARDSKISAAS RFCRSLVG.G ISKSPEMNRV PGVYQLCLKD GSDSGSPGAI
 MmKIF1A  SRDAKLP.AS RSIRNLFGSG SLRATEGNRV TGVYELSLCH VADAGSPGMQ
 MmKIF1B  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
MmKIF1Bb  SRDAKIS.PP RSLRNLFGSG YSKSPDSNRV TGIYELSLCK MADTGSPGMQ
 RnKIF1D  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
 HsKIF1C  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
  HsATSV  SRDAKLP.AS RSIRNLFGSG SLRASESNRV TGVYELSLCH VADAGSPGMQ
NcKin332  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~

          1651
  NcKIF1  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
  UmKin3  R.....LDTG SKYVRGQEML GGWKARGVSL VGDHAALVQR ERRRAEVEGV
DdUnc104  SSPKSGRSSN TTSSSSGGGG GGGRKRSSTI VEVKVKEVPS SALLKE.DET
Ceunc104  RRQRRVLDTS SAYVRGEENL GQWRPRGDSL IFEHQWELEK LTRLQQVERV
 MmKIF1A  RRRRRVLDTS VAYVRGEENL AGWRPRSDSL ILDHQWELEK LSLLQEVEKT
 MmKIF1B  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
MmKIF1Bb  RRRRKVLDTS VAYVRGEENL AGWRPRGDSL ILEHQWELEK LELLHEVEKT
 RnKIF1D  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
 HsKIF1C  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
  HsATSV  RRRRRVLDTS VAYVRGEENL AGWRPRSDSL ILDHQWELEK LSLLQEVEKT
NcKin332  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~

          1701
  NcKIF1  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
  UmKin3  RATLKGRSAM MRNVDDANAE SKEELAARVV AVWQRAVRDS KVGVVIGVQP
DdUnc104  SGYLKKKSAF KEEWKPRWFV FKKPYLYYSH NQKDTHKLKK IDLTNSSVAI
Ceunc104  RLFLRLRDRL ..KGKKNKGE ARTP...... .......... .......VSP
 MmKIF1A  RHYLLLREKL .ETTQRPGPE VLSP.ASSED SESRSSS... ........GA
 MmKIF1B  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
MmKIF1Bb  RHFLLLRERL GDSVPKSLSD SLSP.SLSSG TLSTSTSISS QISTTTFESA
 RnKIF1D  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
 HsKIF1C  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
  HsATSV  RHYLLLREKL .ETAQRPVPE ALSP.AFSED SESHGSS... ........SA
NcKin332  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
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          1751
  NcKIF1  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
  UmKin3  STNAASAGGT TCKTGADGLV GMFAAPSAVD GTNGLGIDNL SASSSPAKIE
DdUnc104  TQDEVPFGFA IIQLRRVWLL QANSVEDRDK WVQTLDPLRK VTELKDEELR
Ceunc104  CDPVCAI.PE SIKLD...EK DKGIVGKVLG LIRRKIPMNK DPPTGNKAQE
 MmKIF1A  SSPLSAEGQP S.PLEAPNER QRELAVKCLR LLMH..TFNR EYTHSHVCIS
 MmKIF1B  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
MmKIF1Bb  ITPSESSGYD SADVESLVDR EKELATKCLQ LLTH..TFNR EFSQVHG..S
 RnKIF1D  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
 HsKIF1C  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
  HsATSV  SSPLSAEGRP S.PLEAPNER QRELAVKCLR LLTH..TFNR EYTHSHVCVS
NcKin332  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~

          1801
  NcKIF1  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
  UmKin3  RTRSTWSSTA PAPAPAPSAP AAPAA.LTAI VALLPRTATT SHRGYLWIPL
DdUnc104  TAKQQIEKSS SQLDQIKSQL QTGQQIVLAK QKEIEELTNT ISQLQLEKEI
Ceunc104  LSDESGSN.S IT.......S PVSDK.SLIK SSRSSDLLCR QKSKSDQNL.
 MmKIF1A  ASESKLSEMS VTLMRDPSMS PLGAA.TLTP SSTCPSLIEG RYGATDVRTP
 MmKIF1B  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
MmKIF1Bb  ISDCTWSDVS .PIGRDPSVS SFSSS.TLTP SSTCPSLVDS RSSSMDQKTP
 RnKIF1D  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
 HsKIF1C  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
  HsATSV  ASESKLSEMS VTLLRDPSMS PLGVA.TLTP SSTCPSLVEG RYGATDLRTP
NcKin332  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~

          1851
  NcKIF1  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
  UmKin3  ETITDTWVRR FLVLRRPFLH IYE..SNAQV DEVMVINVEA V.RVEYDENT
DdUnc104  NTQQFDGLRD EIQNRDEELE QYKSQQSQKI NQLSGQVNKL ENVTQEKELT
Ceunc104  ......ASND DIVDNLGGM. .KRSLSGSRI LQLN.....I L.VPEVLEER
 MmKIF1A  QPCSRPASP. .EPELLPELD SKKTPSPVRA TETEKEPQRL L.VPDIQEIR
 MmKIF1B  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
MmKIF1Bb  EANSRASSPC QEFEQFQIVP TVETPYLARA ...GKNEFLN L.VPDIEEVR
 RnKIF1D  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
 HsKIF1C  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
  HsATSV  QPCSRPASP. .EPELLPEAD SKKLPSPARA TETDKEPQRL L.VPDIQEIR
NcKin332  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~

          1901
  NcKIF1  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
  UmKin3  ERLLGKQNVF AVYTANNSYF FQADSDKDRQ VWMKLLDGSY NGDAGHVFC*
DdUnc104  IGSLSSTLNN TNQIIELINE QSKSYKNVAE MEIESLRDET TQLRETSQLL
Ceunc104  VGVVVSKKGY MNFLEEKTQG WTRRWVIVRR PYILLFRDDR DLVIRGIINL
 MmKIF1A  VSPIVSKKGY LHFLEPHTAG WAKRFVVVRR PYAYMYNSDK DTVERFVLNL
 MmKIF1B  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
MmKIF1Bb  AGSVVSKKGY LHFKEPLSSN WAKHFVVVRR PYVFIYNSDK DPVERGIINL
 RnKIF1D  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
 HsKIF1C  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
  HsATSV  VSPIVSKKGY LHFLEPHTSG WARRFVVVRR PYAYMYNSDK DTVERFVLNL
NcKin332  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
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          1951
  NcKIF1  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
  UmKin3  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
DdUnc104  ANRLKECRSS IQSAESLLSE RDLEITQLKA LLTQQEESSG ITSLNLKNLQ
Ceunc104  ANARIEHSED QQAMVKVPNT FSVCTNQRGF LMQMMPGDEM YDWLYAINPL
 MmKIF1A  STAQVEYSED QQAMLKTPNT FAVCTEHRGI LLQANSDKDM HDWLYAFNPL
 MmKIF1B  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
MmKIF1Bb  STAQVEYSED QQAMVKTPNT FAVCTKHRGV LLQALNDKDM NDWLYAFNPL
 RnKIF1D  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
 HsKIF1C  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
  HsATSV  ATAQVEYSED QQAMLKTPNT FAVCTEHRGI LLQAASDKDM HDWLYAFNPL
NcKin332  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~

          2001
  NcKIF1  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
  UmKin3  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
DdUnc104  SDQTMKQGQI DILSKTVQQS TATIQNISSQ LDSTTKASDS KDEQITSINS
Ceunc104  MAGQMKLHGN QNGTTLKSPT SSSSIAAS~~ ~~~~~~~~~~ ~~~~~~~~~~
 MmKIF1A  LAGTIRSKLS RRRSAQMRV~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
 MmKIF1B  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
MmKIF1Bb  LAGTIRSKLS RRCPSQPKY~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
 RnKIF1D  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
 HsKIF1C  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
  HsATSV  LAGTIRSKLS RRRSAQMRV~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
NcKin332  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~

          2051
  NcKIF1  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
  UmKin3  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
DdUnc104  AYKDESDRLK DQTTQLNSLT TNLRQQMRSL EQTHLQQKET SASDQKTLLL
Ceunc104  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
 MmKIF1A  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
 MmKIF1B  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
MmKIF1Bb  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
 RnKIF1D  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
 HsKIF1C  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
  HsATSV  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
NcKin332  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~

          2101
  NcKIF1  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
  UmKin3  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
DdUnc104  LLHDMEQGLT RASQTITDQS AQVTVLKKQL EDSKKSNEQL PTVEKQLSLM
Ceunc104  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
 MmKIF1A  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
 MmKIF1B  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
MmKIF1Bb  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
 RnKIF1D  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
 HsKIF1C  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
  HsATSV  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
NcKin332  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
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          2151
  NcKIF1  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
  UmKin3  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
DdUnc104  KDRLIQSENQ LIDRECENTI LSDKLKLWEE EIKIKDSKLS LLENNVKEVR
Ceunc104  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
 MmKIF1A  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
 MmKIF1B  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
MmKIF1Bb  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
 RnKIF1D  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
 HsKIF1C  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
  HsATSV  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
NcKin332  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~

          2201
  NcKIF1  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
  UmKin3  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
DdUnc104  AEYANGMAFS REFSQHHTDS GSISGKFNRR SKQISAEEQM ETLRESSIAH
Ceunc104  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
 MmKIF1A  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
 MmKIF1B  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
MmKIF1Bb  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
 RnKIF1D  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
 HsKIF1C  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
  HsATSV  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
NcKin332  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~

          2251
  NcKIF1  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
  UmKin3  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
DdUnc104  QSHNAFLNSQ IQRLETEMRT QEKVYSDTIQ RIKKDLQQRN QQNIAFMKHQ
Ceunc104  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
 MmKIF1A  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
 MmKIF1B  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
MmKIF1Bb  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
 RnKIF1D  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
 HsKIF1C  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
  HsATSV  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
NcKin332  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~

          2301
  NcKIF1  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
  UmKin3  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
DdUnc104  VGDEIVKKME DVTASMEILK KKYFVSLVVA AKLQNAMMGN ICNVDAYELY
Ceunc104  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
 MmKIF1A  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
 MmKIF1B  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
MmKIF1Bb  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
 RnKIF1D  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
 HsKIF1C  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
  HsATSV  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
NcKin332  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
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          2351
  NcKIF1  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~
  UmKin3  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~
DdUnc104  EQSVVEHILD QDQWPNWIAQ TISTQNKHL
Ceunc104  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~
 MmKIF1A  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~
 MmKIF1B  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~
MmKIF1Bb  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~
 RnKIF1D  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~
 HsKIF1C  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~
  HsATSV  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~
NcKin332  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~
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CURRICULUM VITAE

Personal Details

Name:	Michaela Hartel
Date and Place of Birth:	7th August 1971, Berlin, Germany
Nationality:	German
Marital Status:	Single

Education and Qualifications

1978 - 1988	Primary and Secondary School, Leaving Certificate: Very Good
1988 - 1990	Extended Secondary School, Abitur (University Entrance Qualification): Very Good
1990 - 1992	Attendance at language-courses at the Humboldt-University Berlin (Medical Terminology, French, English)
1991 - 1992	Studies of Geophysics at the Free University Berlin
1992 - 1999	Medical studies at the Humboldt-University Berlin, Clinical electives in Berlin, Winchester (UK), Oxford (UK) and Toronto (Canada)
1999	Third State Examination of the medical studies (final examination)
1999 - 2003	Doctoral thesis in the laboratory of Prof. Dr. Manfred Schliwa at the Ludwig-Maximilians-University Munich. Title: „Molecular Cloning and Functional Studies of *Neurospora crassa* KIF1, a New Member of the UNC-104/KIF1A Family of Kinesin-Like Proteins“

Grants

1994 - 1999	Scholarship by the Reemtsma-Begabtenförderungs-Werk
Jul. - Aug. 1995	Grant by the German Academic Exchange Service (DAAD) for the attendance of an English-language programme at the Michigan State University/East Lansing, USA