The Aspergillus nidulans Kinesin-3 Tail Is Necessary and Sufficient to Recognize Modified Microtubules

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Abstract

Posttranslational microtubule modifications (PTMs) are numerous; however, the biochemical and cell biological roles of those modifications remain mostly an enigma. The Aspergillus nidulans kinesin-3 UncA uses preferably modified microtubules (MTs) as tracks for vesicle transportation. Here, we show that a positively charged region in the tail of UncA (amino acids 1316 to 1402) is necessary for the recognition of modified MTs. Chimeric proteins composed of the kinesin-1 motor domain and the UncA tail displayed the same specificity as UncA, suggesting that the UncA tail is sufficient to establish specificity. Interaction between the UncA tail and alpha-tubulin was shown using a yeast two-hybrid assay and in A. nidulans by bimolecular fluorescence complementation. This is the first demonstration of how a kinesin-3 motor protein distinguishes among different MT populations in fungal cells, and how specificity determination depends on the tail rather than the motor domain, as has been demonstrated for kinesin 1 in neuronal cells.

Introduction

The microtubule (MT) cytoskeleton is assembled from alpha, beta-tubulin heterodimers. In addition, multiple isoforms and posttranslationally modified tubulins (PTMs) are known [1]. For instance, certain neuronal cells use alpha-tubulin where the C-terminal tyrosin is cleaved (detyrosinated alpha-tubulin) [2]. Other modifications comprise acetylation, polyglutamylation, or phosphorylation [1]. How posttranslational modifications affect specific functions is largely unknown, although there is increasing evidence that modifications act as “traffic signs” for microtubule-dependent motor proteins [3]. Recently, it was shown that differences in the ratio between tyrosinated and detyrosinated alpha-tubulin in axons and dendrites confer directional cues for kinesin-1-dependent transport in axons [2,4].

In lower eukaryotes only some alpha-tubulin modifications were identified and it appears that certain modifications arose at different times during evolution [1]. There is evidence that detyrosinated or otherwise modified MTs exist in the filamentous fungus A. nidulans. In two-dimensional gels four protein spots were identified as alpha-tubulin although only two genes are found in the A. nidulans genome. The same situation was found for beta-tubulin [5,6]. Further evidence came recently from a study related to the kinesin 3 motor UncA [7]. Kinesin-3 motors contain the conserved motor domain, a FHA domain (forkhead homology-associated domain) involved in phosphorylation dependent protein-protein interactions, signaling pathways and the regulation of kinesin motors and a PH domain (Pleckstrin homology domain) at the carboxy terminus for cargo binding [8]. In A. nidulans and Ustilago maydis kinesin-3 is involved in vesicle trafficking, and deletion of the gene causes a reduction of the growth rate [7,9].

Most surprisingly, UncA<sup>rigor</sup> did not decorate all microtubules in a hyphal compartment of A. nidulans but only a subpopulation consisting of modified alpha-tubulin. An antibody against tyrosinated alpha-tubulin did not recognize the MT decorated by UncA<sup>rigor</sup>. This suggested that the modified MT might consist of detyrosinated alpha-tubulin [7]. However, direct biochemical evidence is not yet available. The exact cargo of UncA also remains to be defined. In N. crassa the motor is involved in mitochondrial distribution and in U. maydis in endosome trafficking [9,10]. In A. nidulans there is evidence that UncA is involved in endosome movement and that endosomes are involved in polarized growth [11,12]. We were meanwhile able to isolate vesicles associated to the UncA motor and are currently analyzing the protein content (own unpublished data).

Fascinating questions refer to the generation and maintenance of different MT populations, their different biological functions and the mechanism of motor-preference for one or the other MT population. Here, we present first evidence of how a kinesin-3 motor protein distinguishes between different MT populations in A. nidulans, and, surprisingly, how a short region in the tail of kinesin 3 is important for “traffic sign” recognition.

Results

The tail of UncA is necessary for microtubule specificity

The kinesin-3 motor UncA binds preferentially to modified microtubules (MTs) [7]. This was most obvious when a rigor mutation was introduced into the motor protein: a GFP-UncA<sup>rigor</sup> fusion protein labeled mainly one bundle of MTs per compartment, whereas other kinesins, mutated and tagged in the same way, labeled several MTs. In order to identify the region that

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confers posttranslationally modified MT specificity, we performed a deletion analysis, starting from a full-length GFP-UncA^{rigor} version. Constructs shorter than 1316 amino acids bound to several MTs, whereas proteins longer than 1402 amino acids displayed specificity for modified MTs (Fig. 1A–C). From these results we concluded that the region between amino acid 1316 and 1402 is involved in modified MT selection. All eight different truncation constructs of UncA^{rigor} were tagged at the N-terminus with GFP, and expressed under the control of the uncA promoter in an uncA-deletion strain. The UncA tail region was compared to several kinesin-3 proteins, from fungi to humans, and showed several highly conserved residues (Fig. S1). In contrast, similar work in rat hippocampal neurons with kinesin 1 revealed a short region in the motor domain, designated the beta5-LB Region, required for specificity [2]. Several residues within this region were essential for discrimination between tyrosinated and detyrosinated MTs; however, the corresponding region in UncA resembled regions in kinesins, which do not prefer detyrosinated MTs (data not shown).

Deletion analysis also revealed that the neck linker (amino acids 379–386), a mechanical element that has been shown to connect the motor domain with cargo and kinesin partner heads [13], was necessary for MT binding of UncA (Fig. 1D). Constructs only harboring the motor domain (379 amino acids), including the predicted MT-binding motif, were unable to bind MTs in vitro and localized to the cytoplasm; these results corroborate findings for Caenorhabditis elegans kinesin 3 (Unc104). This kinesin 3 undergoes concentration-dependent dimerization in vitro as a result of two short helical domains that are directly C-terminal to the neck linker [14]. The neck linker of mouse KIF5C (kinesin 1) can functionally and structurally replace one of its motor heads to generate non-processive motility [17]. The data of Adio and Woehlke confirmed that the neck domain is required for dimerization and is essential for NcKin3 function: the neck linker is an element that connects the motor domain to the cargo, or to another motor domain in the case of kinesin dimers, indicating that this element is essential for motor function.

Recently, Huckaba et al. showed that Drosophila kinesin 3, Khc-75, exists in vitro and in vivo in an equilibrium between monomer and dimer, is enriched at the ends of MTs, and is recruited to Rab3-containing vesicles [16]. In contrast, kinesin 3 from Neospora crassa, NcKin3, was shown to be dimeric, but inactivates one of its motor heads to generate non-processive motility [17]. The data of Adio and Woehlke confirmed that the neck domain is required for dimerization and is essential for NcKin3 function; the absence of the neck altered the kinetic cycle fundamentally [18].

In order to further characterize the function of the 86 amino acids in the A. nidulans UncA tail and concurrently preserve as much of the UncA protein as possible, we deleted only this section of amino acids in the full-length protein expressed under the native promoter. This construct was transformed into an uncA-deletion strain in order to exclude homologous integration events into the uncA locus. We observed loss of specificity in this modified motor protein, confirming the role in specificity determination for modified MTs in the tail of UncA (Fig. 1E). Furthermore, we deleted the 86 amino acids in the original strain SNZ14 (alcA::GFP::uncA^{rigor}), which has been previously used in localization studies [7]. This was achieved using a complementation strategy into the GFP-UncA^{rigor} strain, with homologous integration in the uncI locus (SNZ14) to exclude effects due to different protein expression levels. In full support of our hypothesis, UncA lacking the 86 amino acids decorated all MTs (data not shown). Similar expression levels of the GFP-UncA proteins in the two strains (SNZ14 and SCoS124) were confirmed by Western blot analysis (Fig. 1F). In order to test the functionality of the UncA protein lacking the 86 aa (SCoS75), colonies of this strain were compared to wildtype and to a UncA-deletion strain. The fact that the colonies of SCoS75 showed the same compact colony phenotype, suggests that the 86 aa are required for function of UncA (Fig. 1G). An alignment of this stretch between different fungi and higher eukaryotes revealed many conserved amino acids in this region (Fig. 1H). In addition, this region is characterized by a positive charge (Fig. 1I).

In order to exclude a potential contribution of other domains in UncA to the determination of MT specificity, the FHA domain and the two coiled regions between the FHA and PH domains were deleted in the same way as the 86 amino acids: neither of these deletions affected the specificity (Fig. 2). The FHA (forkhead-associated) domain has been shown to be involved in phosphorylation-dependent protein-protein interactions, signaling pathways [19] and regulation of kinesin motors [20].

The tail of UncA is sufficient for microtubule specificity

If the specificity of UncA is determined by the tail region of the motor, we asked ourselves whether the tail is capable of rendering conventional kinesin (KinA) specific for this MT subpopulation. To this end, we fused the KinA motor domain with the tail region of UncA under the control of the uncA promoter (Fig. 3A). Kinesin-1 and Kinesin-3 show different localization patterns in hyphae. Whereas the GFP-UncA fusion protein labels small bidirectional moving spots, which accumulate at the tip (Fig. 3B), KinA shows diffuse labeling of the entire cytoplasm (Fig. 3C). This has been observed before in A. nidulans and in U. maydis. Stable association of KinA with MTs was only observed after depletion of ATP or introduction of a rigor mutation [7,19,22]. Apparently the cytoplasmic concentration of KinA is quite high and association with MTs only temporarily. Furthermore, KinA transports – in addition to vesicles - also dynein towards the MT plus end [23]. These are likely only single molecules or dimers moving along MTs and thus cannot be visualized with normal epifluorescence microscopy of GFP-KinA. The generated chimeric protein KinA-UncA was N-terminally tagged with GFP and transformed into an uncA-deletion strain. Ecotropic integration into the genome - to exclude integration into the kint locus - was confirmed by Southern blot analysis (data not shown). The resultant strain showed vesicle movement and accumulation of the GFP signal at the tip of transformed hyphae (Fig. 3D), and the chimera was able to rescue the uncA-deletion phenotype, suggesting that the fusion protein is biologically active and transports probably UncA cargoes similar to the wildtype (Fig. 3E).

Subsequently to further analyze the specificity of this chimera, we created a strain with the rigor mutation at the ATP binding site of this chimeric motor; interestingly, this strain also displayed impressive specificity for the MT subpopulation (Fig. 3F), indicating that the tail of UncA is sufficient for generating MT specificity. As a further control, another chimeric motor protein was constructed by fusing the UncA^{rigor} motor domain to the tail of KinA. This fusion protein was also tagged with GFP at the amino terminus and expressed under the control of the uncA promoter to guarantee comparable expression levels. A loss of specificity was displayed, and several MTs were labeled (Fig. 3G). Likewise, a chimeric protein of the UncA^{rigor} motor domain and the tail of kinesin 7, KipA, did not show any preference for MT subpopulations (data not shown). Taken together, these findings suggest that the UncA tail is necessary for vesicle selection and MT specificity determination. The UncA motor domain appears only required for movement and can be replaced by the KinA motor domain.

The tail of UncA is able to bind alpha-tubulin

Because the tail of UncA is apparently able to interact with MTs or MT-associated proteins, we tested for interaction between alpha
Figure 1. UncA-deletion analysis reveals that the tail of UncA is involved in specificity determination. (A) Scheme for the UncA-deletion analysis. The number of amino acids is given in front of the truncated proteins. Motor = motor domain containing the lysine-rich loop (K) and a rigor mutation in the P-loop (asterisk); FHA = forhead associated domain; PH = pleckstrin homology domain. The red square indicates a 86 aa amino acid stretch. (B-E) Localization of different UncA truncated versions (as indicated) in the UncA strain SNZ9. UncA proteins were labeled with GFP and expressed under the control of the uncA promoter. Scale bar, 5 μm. (F) Confirmation of expression levels by Western blot analysis of GFP-UncA<sup>308tr</sup> (206 kDa)(SNZ14) and GFP-UncA<sup>324tr</sup> (1316–1402 (194 kDa)(SCoS124). Western blot detection was done with anti-GFP antibodies (1:4000) and anti-rabbit IgG peroxidase conjugated secondary antibodies (1:4000). 285 ng crude protein extract was loaded. (G) Colonies of SNZ9, SCoS75 and wildtype (TN02A3). (H) Alignment of the 86 aa region of UncA orthologues from different fungi and higher eukaryotes. Done with CLC Sequence Viewer 6. See also Figure S1. doi:10.1371/journal.pone.0030976.g001

Taken together, the results suggest interaction between the tail of UncA and alpha-tubulin. Given that the interaction between the two proteins took place in the nucleus in the yeast-two hybrid assay and in the cytoplasm in A. nidulans in the BiFC analysis, suggests rather direct interaction than complex formation with other proteins.

Discussion

Most eukaryotes contain several tubulin-encoding genes that generate alpha-tubulin with slightly different properties. In A. nidulans, for example, two genes encode for α and two for beta-tubulin [5,6,25]. Although deletion of one of the alpha-tubulin genes, tubB, was possible, this strain was subsequently unable to reproduce sexually; this defect was partially overcome via overexpression of tubA. These results demonstrate nicely overlapping but also distinct functions of certain tubulin isoforms; however, this picture becomes further complicated by the fact that a number of different posttranslational MT modifications exist. Already in 1975, Arce et al. reported the posttranslational incorporation of L-tyrosine into alpha-tubulin, indicating the presence of tyrosinated and detyrosinated MT forms [26]. Alpha-tubulin generally ends with the tripeptide EEY, and the tyrosine residue is cyclically removed by carboxypeptidase, then re-added to the chain by tubulin-tyrosine ligase (TTL) [1]. Other possible modifications include acetylation, polyglutamylation, polyglycylation or phosphorylation [1]. In general, little is known about either modifying enzymes or the biological functions of these modifications, although the suppression of tubulin tyrosine ligase and subsequent accumulation of detyrosinated tubulin favors tumor growth in animal models and human cancers [27].

In neurons it was demonstrated that polarized trafficking of kinesin-1-driven vesicle movement is regulated through the balance between tyrosinated and detyrosinated MTs [2]. Since somatodendrites contain tyrosinated alpha-tubulin and the axon contains detyrosinated alpha-tubulin, inhibited binding of kinesin-1 to tyrosinated MTs restricted the transport function to the axons.

Figure 2. Analysis of UncA versions with deletions of the forhead associated domain (FHA)(SCoS61), the pleckstrin homology domain (PH)(SCoS16), and the coiled coils (CC)(SCoS81) region. Hyphae are 3 μm in diameter. doi:10.1371/journal.pone.0030976.g002
In this case, the β5-L8 region in the motor domain was responsible for MT discrimination. In contrast, we show that in *A. nidulans* the 86 amino acid long region in the tail of kinesin-3 UncA was involved in the recognition process of modified, possibly detyrosinated MTs. Interestingly, UncA cargoes appear to be un-involved in MT recognition, since deletion of the PH domain did not alter its specificity. In comparison, there is evidence that mammalian kinesin-1 cargo proteins may be involved in specificity determination in neuronal cells [28]. Unfortunately, no structural data are available yet for the tail of a kinesin-3 motor protein, because only the motor domain and its binding to MTs has been analyzed [29]. In comparison to our data, it was shown in mice that kinesin-3 uses polyglutamylation as a neural molecular traffic sign, although the structural mechanism of the kinesin remained enigmatic [30]. The picture becomes increasingly complex when considering the recent observation that the modification type may switch from detyrosination to acetylation upon polarization of epithelial cells [31]. It will be the challenge for future studies to unravel the exact mechanism(s) of motor proteins for MT discrimination between different MTs, and determine how posttranslational modifications contribute to navigational cues for different motor proteins.

### Methods

#### Strains, Plasmids, and Culture Conditions

Supplemented minimal media (MM) for *A. nidulans* and standard strain construction procedures are described by Hill and Käfer (2001) [32]. A list of *A. nidulans* strains used in this study is given in Table 1. Standard laboratory *Escherichia coli* strains (XL1 blue, Top 10) were used. Plasmids are listed in Table 2. *S. cerevisiae* strains are listed in Table 3.

#### Molecular Techniques

Standard DNA transformation procedures were used for *A. nidulans* [33] and *E. coli* [34]. For polymerase chain reaction (PCR) experiments, standard protocols were applied. DNA sequencing was performed commercially (Eurofins MWG Operon Ebersberg, Germany). DNA analyses (Southern hybridizations) were performed as described [34].

Domain deletion of UncA was achieved using primers with phosphorylated 5'-ends to amplify the entire vector pCoS21, with the exception of the deleted region. The primers used for the FHA mutant were del_FHA_fwd (P-5'-CCC GAG GAA GCA AGG GCT GAA C-3) and del_FHA_rev (P-5'-CTT TTT CGG TGT

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Figure 3. Chimeric kinesin proteins verify that the UncA tail is sufficient for microtubule specificity. (A) Scheme for the creation of chimera of kinesin 1 (yellow), KinA, and kinesin 3, UncA (green). (B) Localization of GFP-UncA (SNZ2) and (C) mRFP-KinA (SC56-NZ). (D) Time lapse of the KinA–UncA chimeric protein (SCoS23). Arrows indicate a moving vesicle. Vesicles also accumulate at the tip of hyphae, similar to the UncA localization. (E) Growth comparison of WT, UncA and the ΔuncA strain complemented with the KinA-UncA chimera (SCoS23). The fusion protein can restore the ΔuncA phenotype. (F) Localization pattern of KinA<sup>ΔPH</sup>-UncA chimera (SCoS24) labeled with GFP in the ΔuncA strain, under the control of the unca promoter. The chimera shows the same specificity as UncA<sup>ΔPH</sup>. (G) In contrast UncA<sup>ΔFHA</sup>-KinA chimera (SCoS44) in ΔuncA, labeled with GFP, under the control of the unca promoter do not label MT subpopulations. Hyphae are 3 μm in diameter.

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Figure 4. The tail of UncA is able to bind to alpha tubulin. (A) Yeast two-hybrid interaction tests with different truncations of UncA to map the interaction site between these proteins. Only the full-length tail region of UncA is able to interact with either of the two alpha tubulins. Transformants were assayed for growth on SD-LW to confirm integration of both constructs (left) and on SD-QDO for nutritional selection for positive interactions (right). The strength of the interaction is shown in the X-$\alpha$-Gal assay. The red square indicates the 86 amino acids region. (B) Bimolecular fluorescence complementation assay with the YFP-C-terminal half fused to the UncA-tail and the YFP-N-terminal half fused to TubA in strain (SCoS126). (C) Subcellular localization of the GFP-UncA-tail in SCoS127. The tail of UncA localizes to vesicles, which moved in antero- and retrograde direction. Hyphae are 3 μm in diameter.

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Table 1. A. nidulans strains used in this study.

| Strain       | Genotype                                                                 | Source                      |
|--------------|---------------------------------------------------------------------------|-----------------------------|
| TN02A3       | pyrG89; argB2, nkuA::argB; pyroA4                                          | Nayak et al. (2006)         |
| SNZ2         | TN02A3 transformed with pAS3 (alcA::sGFP::uncA), pyroA4                   | Zekert et al. (2009)        |
| SNZ9         | TN02A3 transformed with pNZ13 (uncA deletion), pyrG89                       | Zekert et al. (2009)        |
| SNZ14        | TN02A3 transformed with pNZ15 (GFP-UncA<sub>379</sub>), pyroA4             | Zekert et al. (2009)        |
| SCS6-NZ      | TN02A3 transformed with pCS-NZ5 (mRFP-KinA<sub>343-927</sub>), pyrG89       | this study                  |
| SCoS15       | SNZ9 transformed with pCoS35 (uncA<sub>p</sub>::GFP::uncA<sub>379-1-1316</sub>); veA1 | Zekert et al. (2009)        |
| SCoS16       | SNZ9 transformed with pCoS38 (uncA<sub>p</sub>::GFP::uncA<sub>379-1-1402</sub>); veA1 | this study                  |
| SCoS21       | SNZ9 transformed with pCoS25 (uncA<sub>p</sub>::GFP::uncA<sub>379-1-445</sub>); veA1 | this study                  |
| SCoS23       | SNZ9 transformed with pCoS44 (uncA<sub>p</sub>::GFP::kinA1--348::uncA373--1630); veA1 | this study                  |
| SCoS24       | SNZ9 transformed with pCoS46 (uncA<sub>p</sub>::GFP::kinA<sub>379-1-348</sub>::uncA373--1630); veA1 | this study                  |
| SCoS44       | SNZ9 transformed with pCoS61 (uncA<sub>p</sub>::GFP::uncA<sub>379-1-379</sub>::kinA343--927); veA1 | this study                  |
| SCoS57       | SNZ14 transformed with pCoS73 (Q1314stop complementation); pyrG89; veA1    | this study                  |
| SCoS58       | SNZ14 transformed with pCoS72 (A1402stop complementation); pyrG89; veA1    | this study                  |
| SCoS61       | SNZ9 transformed with pCoS80 (uncA<sub>p</sub>::GFP::uncA<sub>343-927</sub>without FHA); veA1 | this study                  |
| SCoS62       | SNZ9 transformed with pCoS81 (uncA<sub>p</sub>::GFP::uncA<sub>343-927</sub>without CC2+CC3); veA1 | this study                  |
| SCoS75       | SNZ9 transformed with pCoS75 (uncA<sub>p</sub>::GFP::uncA without 86 aaI); pyrG89; veA1 | this study                  |
| SCoS124      | SNZ14 transformed with pCoS135 (complementation without 86 aaI); pyrG89; veA1 | this study                  |
| SCoS126      | TN02A3 transformed with pCoS155 and pCoS151 (alcA<sub>p</sub>::YFP<sup>C</sup>::uncA<sub>379-1</sub>, alcA<sub>p</sub>::YFP<sup>N</sup>::TubA; veA1 | this study                  |
| SCoS127      | TN02A3 transformed with pCoS156 (alcA<sub>p</sub>::GFP::uncA<sub>379-1</sub>without FHA); pyroA4; veA1 | this study                  |

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Table 2. Plasmids used in this study.

| Plasmid   | Construction                                                                 | Source                      |
|-----------|------------------------------------------------------------------------------|-----------------------------|
| pCR2.1-TOPO | Cloning vector                                                              | Invitrogen                  |
| pCMBlapx   | alcA::GFP, for N-terminal fusion of GFP to proteins of interest; contains N. crassa pyr4 | Efimov et al. (2006)        |
| pGBK7T    | Yeast Two-Hybrid bait vector, Gal4-BD                                        | Clontech                    |
| pGAD7T    | Yeast Two-Hybrid prey vector, Gal4-AH                                       | Clontech                    |
| pNZ5–49   | 1.5-kb uncA(p) fragment in pAS3 with KpnI-EcoRI sites                       | Zekert et al. (2009)        |
| pNZ5–71   | alcA::mRFP::uncAfull length with AscI-Pacl sites in pCMBlapx                 | Zekert et al. (2009)        |
| pNZ5–15   | pAS3 mutagenesis to introduce the G116E mutation in the p-loop of UncA, (UncA rigor) | Zekert et al. (2009)        |
| pCS5–NZ   | pCS2-NZ mutagenesis to introduce the G97E mutation in the p-loop of KinA, (KinA rigor) | Zekert et al. (2009)        |
| pCoS52–55 | uncA::sGFP::uncAfull–1–445 with AscI-Pacl sites in pCMBlapx                  | this study                  |
| pCoS58–54 | uncA::sGFP::uncAfull–1–1316 with AscI-Pacl sites in pCMBlapx                 | this study                  |
| pCoS4–46  | uncA::sGFP::uncAfull–1–1402 with AscI-Pacl sites in pCMBlapx                 | this study                  |
| pCoS4–56  | uncA::sGFP::uncAfull–1–1316–1630 with EcoRI-Pacl sites in pCMBlapx          | this study                  |
| pCoS56–61 | uncA::sGFP::uncAfull–1–1402::kinA343–928 with EcoRI-Pacl sites in pCMBlapx  | this study                  |
| pCoS61–72 | SNZ14 complementation construct A1402stop mutation (AscI-uncA-full-Pacl-pyro-NotI-uncA-RB-Asci) in Topo2.1 | this study                  |
| pCoS73–75 | SNZ14 complementation construct A1316stop mutation (AscI-uncA-full-Pacl-pyro-NotI-uncA-RB-Asci) in Topo2.1 | this study                  |
| pCoS58–85 | uncA tail without PH (951–1497) with EcoRI-Smal sites in pGBK7T             | this study                  |
| pCoS8–86  | uncA tail with PH (951–1631) with EcoRI-Smal sites in pGBK7T                | this study                  |
| pCoS8–87  | uncA::sGFP::uncAfull–390 with EcoRI-Smal sites in pGBK7T                    | this study                  |
| pCoS13–35 | SNZ14 complementation construct without 86 aa (without 1316–1402) (AscI-uncA-full-Pacl-pyro-NotI-uncA-RB-Asci) in Topo2.1 | this study                  |
| pCoS15–11 | alcA::pYFP::tubA1–179th with AscI-Pacl sites in pCMBlapx                     | this study                  |
| pCoS15–15 | alcA::pYFP::uncAfull-length tail with AscI-Pacl sites in pCMBlapx, pyro instead of pyr4 | this study                  |
| pCoS15–16 | alcA::pYFP::uncAfull-length tail with AscI-Pacl sites in pCMBlapx            | this study                  |

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Table 3. S. cerevisiae strains used in this study.

| Strain   | Genotype                                                                 | Source                      |
|----------|--------------------------------------------------------------------------|-----------------------------|
| AH109    | MATα, trp1901,leu2–3,112, ura3–52, his3–200,gal4A, gal80A, lys2, GAL1UAS::GAL1TATA::His3, GAL2UAS::GAL2TATA::Ade2, URA3::MEL1::MEL1TATA::loz2, MEL1 | Clontech                    |
| Y187     | MATα, ura3–52, his3–200, ade2–101, trp1–901, leu2–3, 112, gal4A, met–, gal80A, MEL1, URA3::GAL1UAS::GAL1TATA::loz2 | Clontech                    |
| yCoS1    | Y187 transformed with pCoS82 (tubA in pGAD7T)                              | this study                  |
| yCoS3    | Y187 transformed with pCoS84 (tub8 in pGAD7T)                              | this study                  |
| yCoS4    | AH109 transformed with pCoS85 (uncA tail without PH (951–1497) in pGBK7T) | this study                  |
| yCoS5    | AH109 transformed with pCoS86 (uncA tail with PH (951–1631) in pGBK7T)     | this study                  |
| yCoS6    | AH109 transformed with pCoS87 (uncArigor motor in pGBK7T)                 | this study                  |
| yCoS8    | AH109 transformed with pCoS89 (uncA without motor (352–1631) in pGBK7T)   | this study                  |

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ACT CAA ACC GAT AAA TCC-3; deletion of 80 aa stretch were done using del300_mutation_fwd (P-3-GTG CAG GGG CGG GGT ATT GCA GAG CCTT AAC G-3) and del300_mutation_rv (P-5-GCAT TAA GCT CAA TGG GGG CCA AGA ATC TAC GAG AAT G-3); and del3CC2+3_fwd (P-5-TAC CCA GTG CCA AGA ATC TAC GAG AAT G-3) and del3CC2+3_rev (P-5 AGC AAG CCT ATC GGT ATC CAT C-3). All plasmids were transformed into the ΔuncA strain SN92.

Tagging Proteins with Green Fluorescent Protein (GFP)

In order to create N-terminal GFP fusion constructs of UncA truncations, 1.2 to 4.4-kb long N-terminal fragments of uncA (starting from ATG) were amplified from pNZ-SI71 with the primers uncA_Asc_fwd1 (5-GGGCGCGCCGCCGCACTGCGCGCAGGAGGTGGGT-3) and several PstI reverse primers. The AscI-PstI-digested PCR fragment was cloned into the corresponding sites of pCMB17apx, yielding truncated versions of UncA. To produce UncA N-terminal tagged with GFP under the native promoter, a 1.5-kb fragment of the putative uncA promoter was amplified from genomic DNA with the primers UncA_nat(P)_EcoRI_f2 (5-GGA ATT CTC ATC ACC TAC TGG AGG CGC GCG-3) and UncA_nat(P)_KpnI_rev (5-GCC TAC CTT TGG CCT ATA GCC CAT ACA CC-3), digested with EcoRI and KpnI, and the two fragments were ligated with EcoRI-KpnI-digested pAS3, yielding pNZ- SH4 (alcA promoter replaced with the uncA promoter in pAS3). All plasmids were transformed into ΔuncA strain SN92. Integration events were confirmed by PCR and Southern blot (results not shown).

Creation of uncAig/ and kinAig/ Mutant Alleles

We changed the UncA glycine residue 116 to glutamate by site-directed mutagenesis using the oligonucleotides UncA_P-Loop_Gly_fwd (5-GGG CAG GGT TCG GAG AAG TCT TAC TCG-3) and UncA_P-Loop_Gly_rev (5-CGAGTTA-GACCTC-TCCGACCCGGTCGACC-3), with plasmid pNZ-SI71 as a template, and the QuickChange XL site-directed mutagenesis kit (Stratagene, Heidelberg, Germany); this yielded plasmids for theuncA promoter thisalcAyielding pNZ-SI49 (uncA promoter replacement). For strain generation, anuncA cDNA fragment corresponding to the C-terminal half of UncA (952–1630 amino acids) was amplified and cloned in the pGBKKT vector, which contains the GAL4 DNA-BD and TRP1 marker (BD Clontech). cDNA of tubB from Aspergillus strain T7A23 were amplified and cloned in the pGADT7-Rec vector, which contains the GAL4 DNA-AD and the LEU2 marker (BD Clontech). pGBK7-associated plasmids were transformed in yeast AH109 (mating type MATa), whereas pGADT7-associated plasmids were transformed in yeast Y187 (mating type MATa alpha). The system utilizes two reporter genes (HIS3 and LacZ) under the control of the GAL4-responsive UAS. Beta-galactosidase activity was analyzed using the colony-lift filter assay with X-Gal (3-bromo-4-chloro-3-indolyl-b-D-galactopyranoside (Karl Roth)) as substrate. Interaction was quantified in a X-Gal assay, which detects the activation of the yeast MEL1 gene, a GAL4-regulated gene used in two-hybrid analyses. MEL1 encodes the secreted enzyme alpha-galactosidase, which hydrolyzes colorless X-Gal into a blue end product.

Supporting Information

Figure S1 Alignment of seven kinesin 3 proteins. The alignment was done using CLC Sequence Viewer 6 with standard settings. (PDF)

Author Contributions

Conceived and designed the experiments: RF CS NZ. Performed the experiments: CS NZ. Analyzed the data: CS NZ. Contributed reagents/materials/analysis tools: RF CS NZ. Wrote the paper: RF CS NZ.

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