# Kinesin and dynein mutants provide novel insights into the roles of vesicle traffic during cell morphogenesis in *Neurospora*

Stephan Seiler*, Michael Plamann[†] and Manfred Schliwa*

**Background:** Kinesin and cytoplasmic dynein are force-generating molecules that move in opposite directions along microtubules. They have been implicated in the directed transport of a wide variety of cellular organelles, but it is unclear whether they have overlapping or largely independent functions.

**Results:** We analyzed organelle transport in kinesin and dynein single mutants, and in a kinesin and dynein double mutant of *Neurospora crassa.* Remarkably, the simultaneous mutation of kinesin and dynein was not lethal and resulted in an additive phenotype that combined the features of the single mutants. The mutation of kinesin and dynein had opposite effects on the apical and retrograde transport, respectively, of vesicular organelles. In the kinesin mutant, apical movement of submicroscopic, secretory vesicles to the *Spitzenkörper* – an organelle in the hyphal apex – was defective, whereas the predominantly retrograde movement of microscopic organelles was only slightly reduced. In contrast, the dynein mutant still had a prominent *Spitzenkörper*, demonstrating that apical transport was intact, but retrograde transport was essentially inhibited completely. A major defect in vacuole formation and dynamics was also evident. In agreement with the observations on apical transport, protein secretion into the medium was markedly inhibited in the kinesin mutant but not in the dynein mutant.

**Conclusions:** Transport of secretory vesicles is necessary but not sufficient for normal apical extension. A component of retrograde transport, presumably precursors of the vacuole system, is also essential. Our findings provide new information on the role microtubule motors play in cell morphogenesis and suggest that kinesin and cytoplasmic dynein have largely independent functions within separate pathways.

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## Background

Kinesin and dynein are molecular motors that use the energy derived from ATP hydrolysis to move unidirectionally, but in opposite directions, along microtubules [1]. Both motors have been implicated in the directed transport of a broad variety of cellular organelles [2]. To study the cell biology of molecular motors, simple eukaryotic organisms such as fungi [3] offer several advantages due to their easy genetic accessibility. Thus, the deletion of kinesin [4–6] or dynein components [7–9] results in informative phenotypes. Interestingly, these phenotypes are strikingly similar, raising the question of whether kinesin and dynein have overlapping functions. Studies in other cell types suggest that dynein and kinesin interact and cooperate in various cellular processes (for example, see [10–13]) and may even share the same receptor on cytoplasmic membranes [14,15].

To elucidate the role(s) of kinesin and dynein, we generated a kinesin and dynein double mutant of *Neurospora crassa* and undertook a detailed, comparative study of the morphogenetic features and intracellular movements of the double mutant and its parental single mutants. The findings reported here provide new information on the cellular basis of morphogenetic defects caused by mutations in molecular motors and suggest that dynein and kinesin have largely independent functions.

## Results

### Growth characteristics of the double mutant compared with the parental kinesin and dynein single mutants

The morphology of wild-type and mutant hyphae is illustrated in Figure 1, and their growth characteristics are summarized in Table 1. At first glance, mutations in kinesin, cytoplasmic dynein or the the dynein activator complex, dynactin, resulted in a similar phenotype. Cell growth, rate of hyphal extension, branching and cell diameter were all affected in a similar fashion (see also [5,7,9]). Differences existed in the way cells grew (straight in the kinesin mutant, curly or 'ropy' in the dynein mutants); dynein mutants are thus designated *ro* or *ropy* mutants, for example, *ro-1* for the dynein heavy chain

**Figure 1**
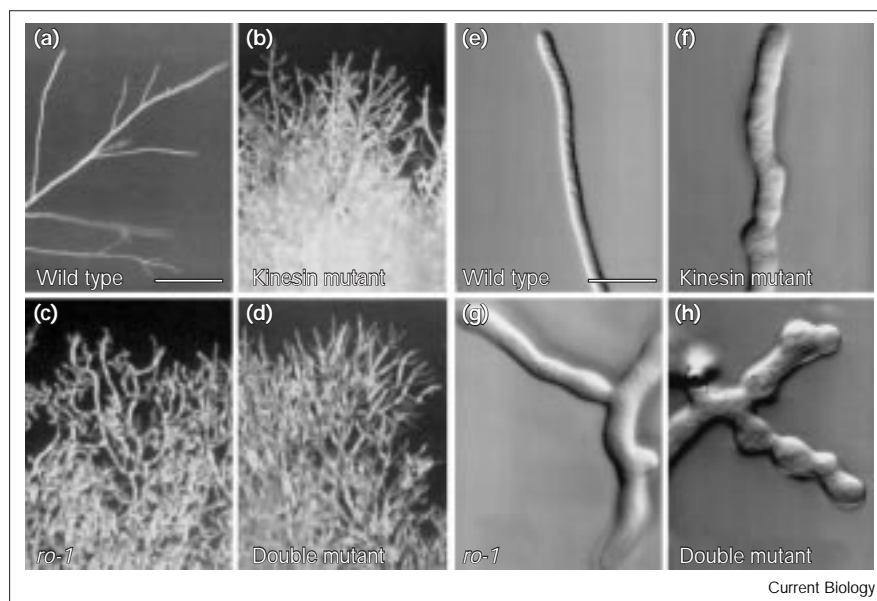


(a–d) Colony morphology and (e–h) hyphal tips of the (a,e) wild type, (b,f) kinesin mutant, (c,g) *ro-1* dynein mutant and (d,h) kinesin and dynein double mutant. Conidia were inoculated onto the centre of agar plates and incubated overnight at room temperature. Photographs are of the colony edge. Note the curly growth pattern of *ro-1* hyphae, which is not observed in the kinesin mutant or in the double mutant. The bar represents 250 µm in (a) and 20 µm in (e).

mutant). Differences also existed in the pattern of septum formation, the frequency of multipolar germlings and the hierarchical organization in hyphal diameter, which was completely lost in the kinesin mutant.

Considering the severe alterations caused by mutating either of the two molecular motors alone, it was remarkable that even the simultaneous loss of both proteins was tolerable. The kinesin and dynein double mutant (*Nkin;ro-1*) combined the characteristic features of the parental single mutants. Interestingly, the curled appearance of the *ro-1* phenotype was suppressed in the double mutant. The cell mass was reduced by half, and there was a dramatic reduction in the rate of hyphal tip elongation, to only 7% that of wild-type hyphae. In the double mutant, nuclei showed the same clustered distribution as in *ro-1*, but still migrated out of the conidia, demonstrating that conventional kinesin is not the motor postulated to be involved in nuclear transport [16].

### *Spitzenkörper* deployment
The *Spitzenkörper* is a characteristic organelle found in the apical dome of hyphal tips that is composed of an

**Table 1**

**Growth characteristics of the motor mutants.**

|  | Kinesin mutant | *ro-1/ro-3* | Double mutant |
|---|---|---|---|
| Growth in 'race' tubes* (% of wild type) | 22% | 22% | 7% |
| Wet weight after 20 h (% of wild type) | 76% | 62% | 51% |
| Cell length | ~1/3 of the wild type | Variable | Variable |
| Hyphal morphology | Straight hyphae; variable diameter, knobby and gnarled appearance | Curled hyphae; variable diameter, appearance ranged from wild-type-like to knobby | Straight hyphae; highly variable diameter, knobby and gnarled appearance |
| Germination | Unipolar/dipolar | Multipolar | Multipolar |
| Nuclear distribution | Slightly clustered | Highly clustered | Highly clustered |
| Vacuole distribution | As in wild type; very dynamic | Often clustered in tip cells; much less dynamic | Often clustered in tip cells; much less dynamic |

*Growth was measured as the rate of extension of a mycelium in long, hollow glass tubes (race tubes; see Materials and methods).
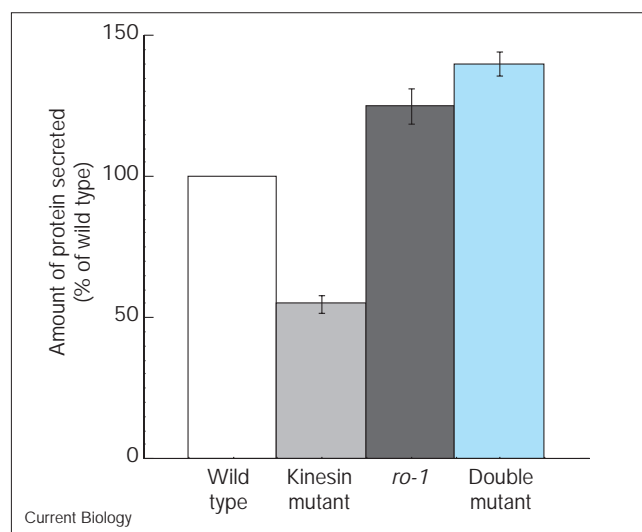
## Figure 2
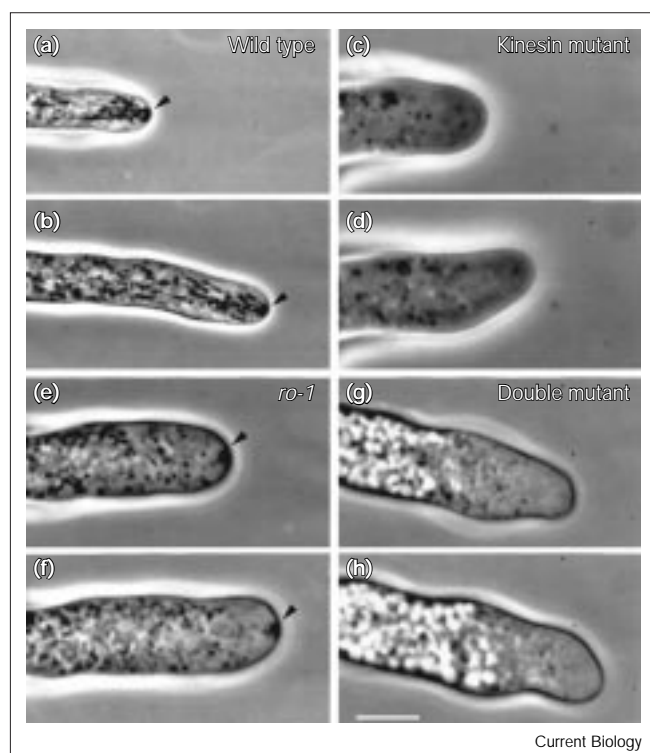


Morphology and hyphal tip growth of the **(a,b)** wild type, **(c,d)** kinesin mutant, **(e,f)** *ro-1* dynein mutant and **(g,h)** double mutant. The time interval between each pair of images was 2 min for the wild type, the kinesin mutant and the dynein mutant, and 22 min for the kinesin and dynein double mutant. The rate of elongation of the hyphal tips shown here was 10 μm/min for the wild type, 3.3 μm/min for the kinesin mutant, 3.6 μm/min for the *ro-1* dynein mutant, and 0.5 μm/min for the double mutant. Note the *Spitzenkörper* in both wild-type and *ro-1* hyphae (arrowheads). The bar represents 10 μm.

## Figure 3



Protein levels in the culture medium of wild-type and mutant hyphae. For each genotype, the mean of three independent experiments is expressed as a percentage of the amount of protein secreted by the wild type.

accumulation of small, presumably secretory vesicles whose presence is linked to cell elongation and apical extension [17,18]. The *Spitzenkörper* of kinesin-deficient hyphae was either much reduced or not readily visible at all, suggesting that conventional kinesin is involved in a step or steps required for the transport of apical vesicles [4–6]. Unexpectedly, the *ro* dynein mutants had a prominent *Spitzenkörper* (Figure 2) even though cell extension of *ro* mutants was as defective as in the kinesin mutant. The double mutant again resembled the kinesin-deficient strain in that a *Spitzenkörper* could hardly be detected by conventional video microscopy.

### Secretion, endocytosis and vacuolation
Hyphal growth requires the secretion and extracellular deposition of cell-wall material; the altered deployment of nascent chitin in the kinesin mutant has already suggested that there are differences in secretion [5]. The deletion of kinesin reduced overall protein secretion by ~50%, as determined by assaying the amount of secreted

protein in liquid cultures. The *ro-1* cultures accumulated slightly more extracellular protein than wild-type cells (Figure 3). This might reflect the fact that secretion only takes place at growing tips [19], which are more numerous in the dynein mutants compared with the wild type. Therefore, the actual rate of secretion per tip could even be overestimated with this assay in the more branched kinesin mutant. This is corroborated by the fact that the double mutant, whose growth pattern was highly disorganized, accumulated even more protein in the medium, and supports the view that long-range transport of vesicles destined for secretion is almost non-existent in this strain.

In contrast, the kinetics of endocytosis, as monitored by the uptake of the vital dye FM 4–64, appeared unaltered in both kinesin and dynein mutants and were comparable to those of wild-type cells (data not shown). There was, however, a dramatic difference in the deployment of the vacuole system (Figure 4). Wild-type and kinesin-deficient hyphae generally vacuolated in older portions of the mycelium only, whereas *ro-1* cells often showed extensive vacuolation near the tip. The latter was also true for the double mutant (data not shown). In both *ro-1* cells and in the double mutant, the dynamics of the vacuolar system was almost completely abolished. Slender tubular extensions frequently seen in hyphae of the wild type and the kinesin mutant rarely occurred in the dynein mutant, and the movement of vesicular vacuoles was curtailed, presumably leading to their accumulation near the hyphal tip.

**Figure 4**
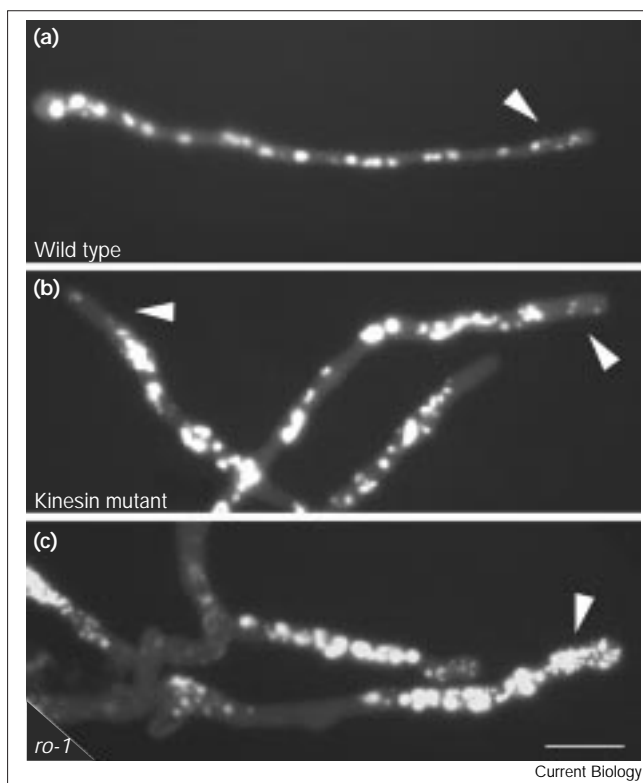


Distribution of vacuole compartments. Both (a) the wild type and (b) the kinesin mutant were characterized by the absence of large vacuoles near the hyphal tip (arrowheads). (c) The *ro-1* dynein mutant had numerous large vacuoles even very close to the hyphal tip (arrowhead). These were extensively clustered and the clusters were unevenly distributed. The double mutant (data not shown) had a similar pattern. In both the wild-type and kinesin-deficient strains, the vacuole system was highly dynamic, often showing tubular extensions not seen in the dynein mutant. The bar in (c) represents 20 μm.

### Organelle movements in the single and double mutants

As both kinesin and dynein are involved in organelle transport in higher eukaryotic cells [2], we used video microscopy to analyze the movement of vesicular organelles in the mutants. To facilitate a quantitative comparison, only growing hyphal tips, which represent the youngest and most active segments of a mycelium, were used for this analysis. All organelles visible by video microscopy, except for nuclei, large vacuoles and mitochondria, were included in the analysis.

The frequency of linear organelle movement was dramatically different between mutant and wild-type tip cells (Table 2). In elongating tip cells of the kinesin-deficient strain, the frequency of organelle movement was only slightly reduced, and was similar to that seen in non-elongating hyphal tips of the wild-type strain. The dynein-deficient mutant *ro-1* showed a dramatic drop in organelle motility; 57% of growing cells showed no linear particle

movements in a straight line across the line of reference within the 30 second time window, and, in the other cells, the frequency of motile events was consistently low. In the double mutant, hardly any particles moving in a linear fashion could be detected at all.

More revealing than just the frequency of organelle movement in the various mutants was the analysis of their directionality (Table 2, lower half). In the wild type, the majority of movements were directed away from the tip, in the retrograde direction. This retrograde bias still prevailed in the kinesin-deficient strain. Remarkably, however, retrograde transport in the dynein mutant was reduced to 1.2% that of wild-type cells, compared with only an approximately twofold reduction in the kinesin mutant. The effect on anterograde transport was relatively moderate. Similar observations were made in the *ro-3* mutant (data not shown), which is defective in the largest subunit of the dynactin complex, p150[Glued] [9]. These findings demonstrate that deletion of cytoplasmic dynein resulted in a specific and virtually complete inhibition of retrograde organelle transport. The fact that anterograde transport of visible organelles was hardly affected in the kinesin mutant suggests the existence of other kinesins powering these movements.

In all mutants, microtubules still extended along the long axis of hyphae as in wild-type cells ([9,20] and our unpublished observations). Mitochondrial motility and positioning was also not affected. These organelles still showed short-range microtubule-dependent excursions in both directions.

### Immunolocalization

To study the transport phenomena in more detail, we used antibodies to localise dynein components and kinesin in *Neurospora*. An antibody against Myc-tagged kinesin gave a specific staining only in strains transfected with a *myc*–kinesin construct. We observed a punctate, vesicular staining pattern throughout the hyphae, superimposed on a more uniform background (Figure 5). The labeled particles were fairly uniform in size. A punctate labeling pattern was also observed with antibodies against *Neurospora* dynein and p150[Glued] (data not shown). The two labeling patterns did not overlap, suggesting that most, if not all, of the kinesin and dynein motors are associated with different vesicle populations.

## Discussion

At first glance, the similar phenotypes of the kinesin and dynein mutants may suggest an involvement of both oppositely directed microtubule motors in related cellular processes. On detailed analysis, however, we found significant differences between the mutant phenotypes. Apical transport, *Spitzenkörper* deployment, retrograde organelle movements, and secretion were all affected in opposite ways by the deletion of either cytoplasmic dynein or

**Table 2**

**Frequencies of particle movement in hyphae grown on slides.**

| Number of moving particles | Wild type; elongating | Wild type; not elongating | Kinesin mutant; elongating | *ro-1*; elongating | Double mutant; elongating |
|---|---|---|---|---|---|
| 0 | 0 | 2% | 2% | 57% | 95% |
| 1–5 | 0 | 36% | 28% | 43% | 5% |
| 6–10 | 14% | 40% | 47% | 0 | 0 |
| 11–15 | 39% | 20% | 23% | 0 | 0 |
| 16–20 | 30% | 2% | 0 | 0 | 0 |
| 21–25 | 12% | 0 | 0 | 0 | 0 |
| 26–30 | 5% | 0 | 0 | 0 | 0 |
| Total number of moving particles | 788 | 362 | 380 | 65 | 7 |
| Number of particles moving towards hyphal tip/number moving in retrograde direction | 221/567 | 174/188 (79%/33%) | 144/236 (65%/42%) | 58/7 (26%/1%) | ND ND |

The frequency of motile events was determined by counting the number of particles crossing a line drawn perpendicular to the long axis of a hyphal tip cell at a distance of about 20 μm from the tip within a time period of 30 sec. In the upper half of the table, the percentage of each type of hypha having the indicated numbers of moving particles is shown. In the lower half of the table, the number of particles moving towards the hyphal tip versus the number moving in the retrograde direction for each hyphal type is shown. These numbers are also expressed as a percentage of the corresponding numbers in wild-type elongating hyphae (indicated by parentheses). These numbers were not determined (ND) in the double mutant because of the low frequency of particle movement. For each hyphal type, 50 hyphae were examined.

kinesin, suggesting that the two molecular motors have largely non-overlapping functions. This conclusion was reinforced by the analysis of the double mutant, which allowed us to assay for possible interactions between two gene products. Whereas proteins acting within the same complex or in the same pathway result in identical phenotypes (for example, see [21,22]), the patchwork of phenotypic expression in the kinesin and dynein double mutant strongly suggests that the two microtubule motors operate within separate pathways.

The roles of kinesin and dynein in membrane movements in other cell types are complex, and their interaction in transport processes is still controversial (for a detailed discussion, see [2]). On the one hand, kinesin and dynein have been proposed to exist as part of a motor complex [10,13]. On the other hand, the comparison of kinesin and dynein deletion mutants in the mouse revealed profound differences between both phenotypes, arguing against the idea that there is a strong interaction between both motors [23,24].

The kinesin and dynein defects reported here were clearly additive in the double mutant. We therefore suggest a model in which the two independent transport systems are both essential for normal polarized cell extension (Figure 6). In this model, kinesin is responsible for the apical transport of small vesicles destined for the *Spitzenkörper* and, ultimately, secretion and cell-wall synthesis. Apical extension would, however, be dependent not only on tip-directed transport, but also on the retrograde movement of certain cell components. The phenotype of the dynein-deficient strain would be directly linked to the striking inhibition of retrograde particle transport, notably of components of the vacuole compartment. In the dynein mutant, vacuoles were found near the hyphal tip, a position in which they never appeared in such abundance in wild-type cells or in the kinesin mutant. The formation of vacuoles in older segments of hyphae has been proposed to build up turgor pressure which is thought to contribute to apical extension [25–27]. As vacuole deployment was affected in the dynein mutant, this mechanism seems to have been defective. The blockage of retrograde transport in the dynein mutant may result in the fusion of organelles targeted to the vacuole/lysosome system with vacuole precursors near the tip, resulting in the formation of extensive apical vacuole compartments. The slow growth observed in *ro* dynein mutants could then be explained as a consequence of slowed membrane cycling. Because the vacuolar compartment of fungi may serve a similar function as the lysosomal compartment of vertebrate cells, and as dynein is involved in movements of the endosomal/lysosomal compartment [23,28], cytoplasmic dynein may have a role that is conserved between the fungal and animal kingdoms.

**Figure 5**
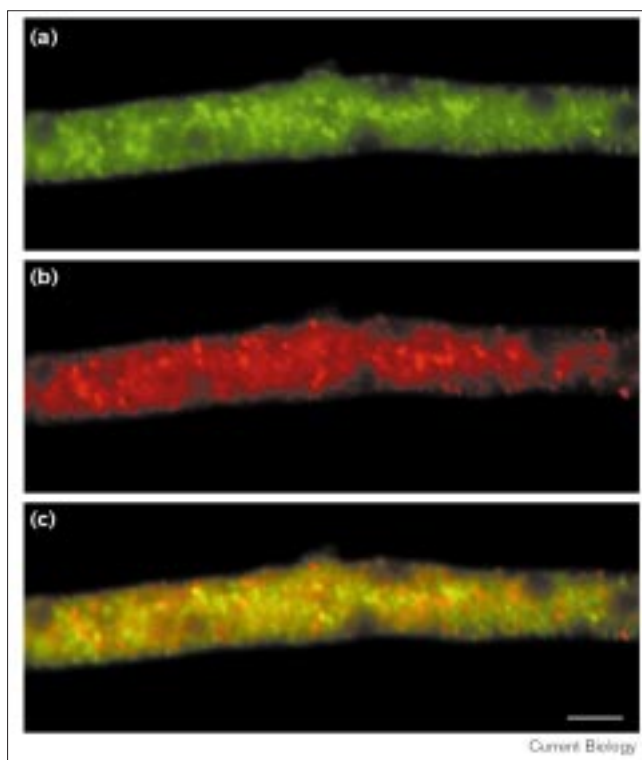


Immunolocalization of **(a)** kinesin and **(b)** cytoplasmic dynein.
**(c)** Superimposition of (a,b). There was no visible overlap between the two punctate labeling patterns, suggesting that the two microtubule motors are associated with different classes of vesicular organelles. The yellow tint in (c) is caused by the diffuse background staining in (a,b). The bar in (c) represents 5 μm.

## Materials and methods
*Strains, growth and handling of* Neurospora
The *N. crassa* strains used here were *ro-1* (B15; FGSC no. 146), *ro-3* [9], *Nkin* null mutant [5], and wild type 74A. The *Nkin;ro-1* double mutant was constructed using the *Nkin* strain as the female (perithecial) strain. Putative double mutants were crossed to the wild type to establish the presence of both the *Nkin* and *ro-1* mutations. Strains were grown in Vogels minimal medium supplemented with 1.5% sucrose. The rate of extension of a mycelium was measured in long, hollow glass tubes (race tubes), half filled with Vogels minimal agar and innoculated at one end with conidia. To measure secreted protein levels, wild-type conidia were innoculated at a concentration of $5 \times 10^6$/ml. The inoculation level of mutant conidia was adjusted accordingly so that all strains reached the same total dry weight after 20 h.

*Light and fluorescence microscopy*
Light and fluorescence microscopy was performed using a Zeiss Axiophot microscope. Intracellular movements were recorded with a Hamamatsu C2400 camera and a Hamamatsu DVS 2000 image-processing system. To visualize hyphal extension, cells were grown in Vogels minimal medium solidified with 15% gelatin directly on a slide. Nuclei were stained with 0.5 μg/ml DAPI. Mitochondria were labeled with rhodamine 123 (10 μg/ml) or $DIOC_6$ (5 μg/ml) added directly to the culture medium. Vacuoles were visualized with 10 μM cell tracker blue (Molecular Probes) added to the medium. For immunofluorescence microscopy, cells were grown overnight on dialysis membranes and fixed for 30 min with 2% formaldehyde and 0.5% glutaraldehyde in

**Figure 6**
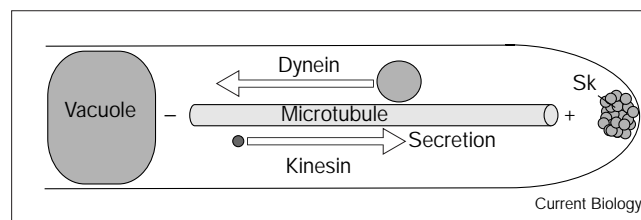


Model of the major transport pathways mediated by dynein and kinesin. In this model, kinesin is responsible for the apical transport of vesicles destined for secretion, whereas dynein transports vacuole precursors in the retrograde direction to support the formation of vacuoles in the basal portion of the hypha. Both motors use a set of microtubules predominantly oriented with their plus-ends near the tip (the plus and minus signs indicate the plus and minus ends of the microtubule). Note that both motors may well be involved in the transport of other cellular constituents but, according to the observations presented here, it is the defects in the transport of secretory and vacuolar components that appear to be chiefly responsible for the mutant phenotypes. Sk, *Spitzenkörper*.

PEM buffer (50 mM K-PIPES pH 6.8, 5 mM $MgSO_4$, 50 mM EGTA). The cell wall was digested for 15 min at room temperature with 1 mg/ml Novozym 234 in a solution containing a proteolysis inhibitor cocktail. Subsequent steps were performed as described [9].

*Myc-tagged kinesin*
A tagged version of kinesin was generated by adding sequences encoding the Myc epitope tag to the 5′ end of wild-type kinesin cDNA. The tagged kinesin moved microtubules in *in vitro* gliding assays at the same rate as did wild-type kinesin and, after transformation into the kinesin-deficient strain, resulted in full rescue of the null phenotype. Antibodies against the Myc epitope recognized only the tagged kinesin in the transfected strain.

*Analysis of organelle movements*
Organelle movements were analyzed in colonies grown on slides. All organelles that were visible using computer-enhanced video microscopy, except nuclei, large vacuoles and mitochondria, were used for the analysis. The frequency of motility events was determined by counting all particles crossing a line drawn perpendicular to the long axis of a hyphal-tip cell at a distance of about 20 μm from the tip within a time period of 30 sec. Only movements longer than 3 μm in one direction were used for the analysis. Care was taken to use only elongating hyphae. In the wild type, non-elongating hyphal tips that were present in the same preparation as elongating tips were included for comparison.

*Supplementary material*
An additional figure showing nuclear distribution of wild-type and mutant hyphae is available at http://current-biology.com/supmat/supmatin.htm.

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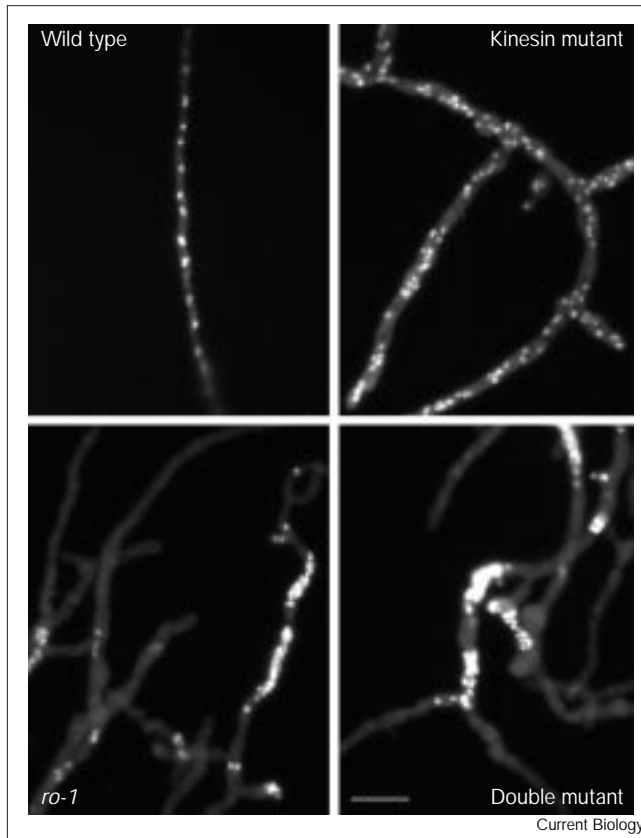
# Supplementary material

## Kinesin and dynein mutants provide novel insights into the roles of vesicle traffic during cell morphogenesis in *Neurospora*

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**Figure S1**



Nuclear distribution visualized by DAPI staining in wild-type and mutant hyphae. Note extensive clustering in *ro-1* and the double mutant, and moderate clustering in the kinesin mutant. The bar represents 20 μm.