Cargo selection by specific kinesin light chain 1 isoforms

Marcin J. Woźniak and Victoria J. Allan

University of Manchester, Faculty of Life Sciences, The Michael Smith Building,

Oxford Road, Manchester M13 9PT, UK

Please address all correspondence to Dr Viki Allan

viki.allan@manchester.ac.uk
Telephone (44) 161 275 5646
Fax (44) 161 275 5082

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Abstract

Kinesin-1 drives the movement of diverse cargoes, and it has been proposed that specific kinesin light chain (KLC) isoforms target kinesin-1 to these different structures. Here, we test this hypothesis using two in vitro motility assays which reconstitute the movement of rough endoplasmic reticulum (RER) and vesicles present in a Golgi membrane fraction. We generated GST-tagged fusion proteins of KLC1B and KLC1D that included the tetratricopeptide repeat (TPR) domain and the variable C-terminus. We find that preincubation of RER with KLC1B inhibits RER motility, whereas KLC1D does not. In contrast, Golgi fraction vesicle movement is inhibited by KLC1D but not KLC1B reagents. Both RER and vesicle movement is inhibited by preincubation with the GST-tagged C-terminal domain of ubiquitous kinesin heavy chain (uKHC), which binds to the N-terminal domain of uKHC and alters its interaction with microtubules. We propose that although the TRR domains are required for cargo binding, it is the variable C-terminal region of KLCs that are vital for targeting kinesin-1 to different cellular structures.

Key words: kinesin/Golgi/endoplasmic reticulum/microtubule/motility

Introduction

Kinesin-1 is the founding member of the kinesin superfamily of microtubule motor proteins. It moves vesicles in squid giant axons (Brady et al., 1990; Hirokawa et al., 1991; Vale et al., 1985) and has been implicated in the dynamics of the ER, Golgi apparatus, secretory vesicles, endocytic structures, mitochondria and mRNP particles (Wozniak et al., 2004). This raises the question as to how one motor can have so many different cargoes, and this may be due to the existence of different motor isoforms. Kinesin-1 comprises two motor subunits (heavy chains, KHCs) and two light chains (KLCs), and in vertebrates, both subunits are encoded by three genes (Junco et al., 2001; Rahman et al., 1999; Xia et al., 1998). In most non-neuronal vertebrate cells, one heavy chain isoform, KIF5B or uKHC, is expressed along with KLC1 and KCL2. There are many different splicing isoforms of KLC1 (Gyoeva et al., 2000; Khodjakov et al., 1998; McCart et al., 2003), although only one KLC isoform type is present in any given kinesin-1 molecule (Gyoeva et al., 2004).

Both heavy and light chains have been suggested to play a role in attaching kinesin-1 to cargo. The C-terminal region of KHC interacts with a number of proteins, and in fungi such as *Neurospora crassa*, which lack light chains altogether, this region is sufficient to bind cargo (Seiler et al., 2000). A variety of proteins interact with the KHC C-terminal domain, between amino acids 813-963, while the KLCs interact with amino acids 771-813 (Adio et al., 2006). Importantly, both the KLCs and most potential cargo molecules can bind simultaneously to KHC (Diefenbach et al., 2002; Huang et al., 1999). An exception to this model is milton, which competes with KLCs for binding to the same KHC domain (Glater et al., 2006).

KLCs are important components of kinesin-1 in higher eukaryotes, as loss of KLC function leads to severe defects in neurons and eventually to death (Gindhart et al., 1998; Rahman et al., 1999). One possible role for KLCs is to allow the inhibition of KHC function

when kinesin-1 is not attached to cargo (Verhey et al., 1998). Native kinesin-1 can exist in a folded, inactive state where the KHC C-terminal domain interacts with the neck region close to the motor domain of KHC, so inhibiting its ATPase activity (Coy et al., 1999; Friedman and Vale, 1999; Hackney et al., 1992; Hackney and Stock, 2000; Seiler et al., 2000; Stock et al., 1999) and its ability to move along microtubules (Coy et al., 1999). The presence of light chains has been proposed to allow kinesin-1 to switch between the inactive, folded state when it is free in the cytosol, and the extended, active form when it is attached to cargo (Verhey et al., 1998).

KLCs interact with a number of potential cargo molecules. KLCs contain an N-terminal coiled-coil domain, termed the heptad repeat, that binds to KHC. Upstream of this region is a short conserved stretch that interacts with the glycogen synthase kinase 3-binding protein GBP/Frat (Weaver et al., 2003). However, the most important region for cargo binding is thought to be a domain C-terminal to the heptad repeat that contains five tetratricopeptide repeats (TPR), which bind to a wide range of proteins (Adio et al., 2006). Consistent with this idea, an antibody to the TPR domain inhibits kinesin-1-driven motility in squid axoplasm by releasing kinesin-1 from the membrane (Stenoien and Brady, 1997). The TPR domains in the various KLC isoforms are almost identical (Rahman et al., 1998), and therefore both KLC1 and 2 would be expected to interact equally with all the cargo molecules identified so far. In contrast, there is considerable variability in the very C-terminal region of KLCs, both between the KLC1 and 2 genes, and within KLC1 populations, due to alternate splicing (McCart et al., 2003). It has been suggested that this C-terminal region is important for targeting kinesin-1 to different cargoes, since KLC1B or C were enriched on mitochondria (Khodjakov et al., 1998) while KLC1D or E were located on the Golgi apparatus (Gyoeva et al., 2000). Furthermore, the C-terminus of KLC2 was shown to be important for kinesin-1 binding to axonal vesicles, since phosphorylation of serine residues in the very C-terminus of

KLC2 leads to release of kinesin-1 from the membrane via the action of Hsc-70 (Morfini et al., 2002; Tsai et al., 2000). The only protein identified so far that interacts with the C-terminus of KLCs is the 14-3-3η protein, which binds to KLC2 when it is phosphorylated on Ser575 (Ichimura et al., 2002).

Here, we aimed to investigate the role of different KLC isoforms in kinesin-1 function. Using in vitro motility assays, we followed the kinesin-1-driven movement of RER membranes, and of vesicles present in a Golgi membrane fraction. We show that KLC1 isoform-specific C-terminal domains play an important role in this process.

Results

Golgi and ER membranes have specific KLC splicing variants

Different KLC1 isoforms, generated by alternate splicing (fig. 1A), have been proposed to target kinesin-1 to specific cargoes (Gyoeva et al., 2000). We decided to use in vitro assays for kinesin-1-driven membrane movement to test the role of specific KLC1 isoforms in kinesin-1 function on different organelles.

Our previous work showed that there is plentiful uKHC in a rat liver Golgi fraction (Robertson and Allan, 2000). As expected, uKHC was also present in a rat liver RER fraction (fig. 1B, upper panel). Reblotting the same nitrocellulose membranes with an antibody (KLC_{ALL}) that recognises all KLC forms (Stenoien and Brady, 1997) revealed that the RER and Golgi membranes contain KLC proteins with different molecular weights (fig. 1B). The RER fraction had a single KLC band, while the Golgi fraction contained one major and two minor bands, in keeping with the RER fraction being more homogeneous than the stacked Golgi fraction (Allan and Vale, 1991; Leelavathi et al., 1970). Based on the size differences, the large isoform present in the Golgi fraction could be KLC1D or E, or KLC2. The RER fraction band could correspond to any of the smaller KLC1 isoforms (A, B, C and F: fig. 1A).

Based on the relative mobilities of the KLC isoforms present in the RER and Golgi fractions, coupled with the published observations that KLC1D/E is associated with the Golgi apparatus (Gyoeva et al., 2000), we decided to test the effects of KLC1B and D, and KLC2, using functional assays. GST-fusion proteins for KLC1B, KLC1D and KLC2 were generated that lacked the heptad repeats that bind to KHCs but contained TPR domain, which is thought to bind to cargo, and the specific carboxyl-terminus (fig. 1C). These fusion proteins are referred to as BTC, DTC and 2TC throughout. In addition, we prepared a carboxyl-terminal

fragment of rat uKHC (aa 771 – 963), which included the KLC-binding domain (fig. 1C). The Coomassie stained purified proteins are shown in fig. 1D.

Exogenous KLC1B, but not KLC1D or KLC2, inhibits motility of RER membranes

Interphase cytosol obtained from *Xenopus laevis* eggs promotes microtubule-based motility of both ER and Golgi membranes isolated from rat liver (Allan and Vale, 1994; Allan and Vale, 1991; Robertson and Allan, 2000). The movement can be analysed in real time using video enhanced differential interference contrast microscopy (VE-DIC). The motility is MT-based, since cytochalasin D is added to prevent actin polymerisation. Virtually no movement occurred in the absence of cytosol (supplementary fig. 1A,B).

When the RER fraction is combined with cytosol, membrane tubules extend along microtubules and fuse with each other to form an extensive two-dimensional network (Allan and Vale, 1994; supplementary fig. 1C). The fusing tubules form three-way junctions and counting these junctions provides a simple indication of the extent membrane tubule movement (Allan, 1995). We used this feature to analyse the effects of the GST-fusion proteins on the motility of RER tubules.

RER membranes were first incubated with GST-KLC fusion protein, or GST as a control, then mixed with *Xenopus* egg cytosol and analysed as described in the Materials and Methods. There was a significant reduction in RER membrane network formation if BTC was used, while incubation with DTC had no effect (fig. 2A; supplementary fig. 1C), suggesting that the inhibition was KLC1 isoform-specific. In support of this conclusion, no inhibition was observed with 2TC, the KLC2-derived fusion protein (fig. 2B, supplementary fig. 1C).

As a further test that RER movement is driven by kinesin-1, we incubated membranes with the C-terminal domain of rat uKHC fused to GST (uKHCct), since the C-terminal segment has previously been shown to inhibit kinesin-1-driven microtubule gliding and

ATPase activity (Coy et al., 1999). While GST alone had no effect (data not shown), uKHCct caused a very strong dose dependent inhibition (fig. 2C; supplementary fig. 1C). Since over 90 % of motility was inhibited with larger amounts of uKHCct, this indicates that kinesin-1 is likely to be the only motor protein that transports rat liver RER.

Motility of vesicles in a rat liver Golgi fraction depends on KLC1D

In contrast to the RER membranes, under the conditions used here the Golgi fraction membranes do not form tubules: instead many vesicles are seen moving along MT in the presence, but not the absence, of cytosol (supplementary fig. 1B). We tested the effect of the KLC and uKHC fusion proteins on this vesicle movement. There was no reduction in vesicle movement if the membranes were preincubated with BTC as compared to GST treated samples (fig. 3A; supplementary fig. 1D). However, DTC reduced the number of moving vesicles by 40 – 50%, depending on the amount of protein used.

The motility of Golgi vesicles was not inhibited completely, which could indicate either that DTC is an inefficient inhibitor, or that another motor protein drives a proportion of the motility. Therefore, we used uKHCct protein, which almost completely inhibited the motility of RER membranes (fig. 2B). However, uKHCct only reduced the number of moving vesicles by about 50% (fig. 3B, compare bars 1, 2, 7 and 8). Moreover, no further inhibition was seen when membranes were preincubated with a mixture of uKHCct and DTC (fig. 3B, bar 4), even when maximally inhibitory levels of uKHCct were used (fig. 3B, compare bars 8 and 9). An intermediate level of inhibition was observed when membranes were treated with uKHCct and BTC together (fig. 3B, bar 3), which is to be expected since the B isoform of KLC does not inhibit motility of the Golgi membranes, and acts to dilute the uKHCct. Finally, as was the case for RER movement, we observed that 2TC has no effect on the motility of Golgi membranes (fig. 3B, compare bars 1, 5 and 6).

Taken together, these data suggest that kinesin-1 is responsible for approximately 50% of vesicle motility in the Golgi fraction and that the carboxyl-terminus of KLC1D is most likely to be involved in the attachment of kinesin-1 to these vesicle membranes.

Cytosolic kinesin-1 is not required for membrane movement

In both motility assays, the presence of *Xenopus* egg cytosol greatly stimulates membrane movement (supplementary fig. 1A,B). Since immunoblotting of *Xenopus* egg cytosol with anti-uKHC reveals plentiful soluble kinesin-1 (fig. 4A), it was possible that *Xenopus* kinesin-1 was being recruited to the membranes to drive the motility we observe, and that recombinant KLC and/or KHC prevented this recruitment. In *Xenopus* there are two uKHC bands, one of which migrates more slowly than the rat liver uKHC, which allowed us to test if *Xenopus* uKHC is recruited to rat liver membranes. As shown in fig. 4A, *Xenopus* egg uKHC remains in the supernatant and no recruitment is observed to either RER or Golgi fraction membranes.

As another approach to test the role of *Xenopus* kinesin-1, we immunodepleted kinesin-1 from *Xenopus* egg cytosol using the SUK4 antibody (Robertson and Allan, 2000), or MYC antibody as a control (Fig. 4B). There was no significant difference in the motility of RER membranes or Golgi fraction vesicles in uKHC or MYC-depleted versus untreated cytosols (fig. 4C). Moreover, the kinesin-1 fusion proteins had similar inhibitory effects in SUK4-immunodepleted cytosol (fig. 4D, E) as they did when untreated cytosol was used (fig. 3).

One possible explanation for the effects of KLC1 and KHC fusion proteins might be that they block the ability of *Xenopus* egg cytosol to activate rat kinesin-1 on the membrane by sequestering an important cytosolic component. To test this possibility, cytosol was pre-incubated with uKHCct, BTC, DTC, or GST coupled to glutathione beads to remove any interacting proteins, and then the depleted cytosol was collected and used in motility assays

as before. This treatment had no effect on the motility of RER tubules or Golgi fraction vesicles (supplementary fig. 1E,F).

From this data, we conclude that neither Golgi fraction vesicle nor RER membrane movement needs *Xenopus* kinesin-1, and that the inhibitory effects of our recombinant proteins likewise do not depend on the presence of *Xenopus* kinesin-1, or on their ability to interfere with the activation of rat kinesin-1 by cytosolic factors.

Kinesin-1 fusion proteins do not release the motor from the membrane

The most obvious explanation for the observed inhibitory effect of the KLC and uKHC fusion proteins is that they cause the release of rat kinesin-1 from the membrane. To test this possibility, RER membranes were incubated with recombinant protein and collected by centrifugation. Neither heavy nor light chains were removed from the membranes by incubation individually with BTC, DTC, uKHCct or GST (fig. 5A). This result could be explained if both KHCs and KLCs contribute to kinesin-1 attachment to membranes, in which case interference with one interaction might not be enough to cause release from the membrane. However, when both KLC1 and uKHC reagents were used together, kinesin-1 still remained membrane-associated (fig. 5A). Similarly, when this experiment was repeated with Golgi membranes, neither subunit was released from the membrane following incubation either with individual fusion proteins (fig. 5B, left panel), or with both uKHC and KLC reagents (fig. 5B, right panel).

Mechanism of uKHCct inhibition

Soluble kinesin-1 is usually in a folded, inactive conformation, where the C-terminal domain of KHC interacts with the heavy chain neck region, leading to inhibition of kinesin-1's motor and ATPase activity. This interaction is thought to be relieved by cargo binding, or by

incubating soluble kinesin-1 at high ionic strength, which generates an extended, active conformation (Coy et al., 1999; Friedman and Vale, 1999; Hackney et al., 1992; Hackney and Stock, 2000; Seiler et al., 2000; Stock et al., 1999). Since the combination of uKHCct and KLC1 fusion proteins did not release kinesin-1 from membranes, we considered the possibility that uKHCct inhibited membrane-bound kinesin-1 by binding directly to the KHC neck, so inhibiting its ATPase activity. To test this, we generated a fusion of the N-terminal half of uKHC (amino acids 1-565) with GFP and expressed it in COS7 cells. Cell lysates were incubated with purified uKHCct bound to glutathione beads, or with beads coated with BTC, DTC or GST (proteins that should not be able to interact with the N-terminal domain of uKHC). The N-terminal of uKHC was seen to interact with uKHCct, but not with the other proteins (fig. 6A), suggesting that uKHCct might indeed inhibit motility by binding directly to the KHC neck.

As a further test of this hypothesis, we examined the effect of uKHCct on the conformation of kinesin-1 using sucrose density gradient centrifugation. We predicted that exogenous uKHCct would compete with the endogenous KHCct for binding to the neck of native kinesin-1, so generating a pool of motor in the extended conformation. When kinesin-1 was solubilised from membranes pre-incubated with GST, BTC or DTC, it migrated in the mid-region of a 5-20% sucrose density gradient (fractions 6/7-10, fig. 6B). In contrast, kinesin solubilised from membranes that pre-treated with uKHCct had a much broader distribution across the gradient, with a proportion being found in the less dense fractions 11-13. This suggests that the incubation with uKHCct had indeed converted some folded kinesin-1 to the extended state.

We then tested the effects of uKHCct and the other fusion proteins on kinesin-1's ability to bind to microtubules in the presence of ATP. RER membranes were pre-incubated with fusion proteins, then solubilised and incubated in the presence of 5 mM ATP, with or

without microtubules (fig. 7A). As a control, membrane lysates were incubated with 400 μM AMP-PNP to induce rigor binding of kinesin-1 to microtubules. In parallel, the same experiment was performed with *Xenopus* egg cytosol (fig. 7B). As expected, both rat RER and *Xenopus* cytosolic kinesin-1 pelleted efficiently with microtubules in the presence of AMP-PNP. Kinesin-1 that had been pre-incubated with BTC, DTC or GST did not bind to microtubules in the presence of 5 mM ATP. However, pre-incubation with uKHCct led to a proportion of kinesin-1 that was sufficiently tightly bound to microtubules in the presence of 5 mM ATP to appear in the microtubule pellet, indicating that the presence of uKHCct slows or inhibits the release of kinesin-1 from microtubules in the presence of ATP. The result is specific to kinesin-1, since kinesin-2 was not affected by the presence of uKHCct (fig. 7B, middle panel). Pre-incubation of rat or Xenopus kinesin-1 with uKHCct did not interfere with the ability of AMP-PNP to promote the binding of kinesin-1 to microtubules (data not shown), further confirming that this recombinant protein does not prevent kinesin-1-microtubule interactions.

Identification of kinesin-1 vesicular cargoes

Although the RER is easy to identify by VE-DIC on morphological grounds, the vesicles present in the Golgi membrane fraction are heterogeneous. Since VLDL particles are easily recognized by EM (Allan and Vale, 1994), we used immuno-EM to determine whether VLDL-containing secretory vesicles possessed kinesin-1. KLC and uKHC antibodies bound to vesicles of 70-200 nm in diameter which were often aggregated together, but did not contain VLDL particles (data not shown). This was confirmed by labelling fixed and permeabilised Golgi membranes (Allan and Vale, 1994) with antibodies to kinesin, apolipoprotein B and albumin (data not shown).

To identify the kinesin-1 positive vesicles, we used an immunofluorescence approach that used unfixed membranes (Bananis et al., 2000; Bananis et al., 2004). Membranes were labelled with H2, a monoclonal anti-KHC antibody, in combination with a polyclonal KHC antibody (HD) or a polyclonal KLC antibody. Although HD, which was raised against *Drosophila* KHC, also recognises other kinesin superfamily members, it reacts most strongly with uKHC (Rodionov et al., 1993). Both polyclonal antibodies showed a similar staining pattern and overlapped with the H2 antibody almost completely (Supplementary fig. 2A). In addition, H2 and HD both gave very similar labelling patterns in normal rat kidney (NRK) cells (Supplementary fig. 3A).

Many kinesin-1-positive vesicles contained p58/ERGIC58 (fig. 8A), a marker for the intermediate compartment/ERGIC that is involved in the transport of material in both directions between the ER and Golgi apparatus. We found occasional overlap on vesicles between kinesin-1 and p115 (fig. 8B), a protein which targets incoming transport vesicles to the Golgi apparatus (Waters et al., 1992). Vesicles within larger clumps of membrane were often labelled with both HD and antibodies to formiminotransferase cyclodeaminase (FTCD) (fig. 8C), a Golgi-localised protein which cycles through the ERGIC (Bashour and Bloom, 1998; Gao et al., 1998; Hennig et al., 1998). There was no colocalisation, however, between HD or H2 and other ERGIC markers such as the KDEL receptor (supplementary fig. 2B), or βCOP (data not shown), or with the COPII component Sec23p (data not shown). In addition, kinesin-1 did not overlap with GM130, a cis-Golgi structural protein that cycles between the late ERGIC and the Golgi apparatus (Marra et al., 2001) (data not shown), nor with Golgin-84 (supplementary fig. 2C).

We also tested whether any of the kinesin-1-positive vesicles were derived from the trans-Golgi or endocytic compartments. While there was no colocalisation between kinesin-1 and TGN38, some vesicles possessed both γ-adaptin and kinesin-1 (fig. 8D). There was no

overlap between kinesin-1 and EEA1 or transferrin receptor (data not shown), whereas a few vesicles were positive for mannose-6-phosphate receptor (M6PR) and kinesin-1 (fig. 8E).

As a control, we labelled NRK cells with the above antibodies. Kinesin-1 antibodies labelled the perinuclear region, and showed general overlap with antibodies to p58/ERGIC58 or FTCD (supplementary figs 3B and 4A,B), γ-adaptin (supplementary fig. 3C) and GM130 (supplementary fig. 4C). This distribution of kinesin-1 in rat cells is consistent with previous work (Marks et al., 1994).

Discussion

Many different cellular processes are orchestrated by the numerous members of the kinesin superfamily. However, even a single motor type, such as kinesin-1, may transport multiple distinct cargoes, but how this is achieved is poorly understood. Here, we provide functional evidence that KLC isoforms determine the specificity of kinesin-1 association with different cargoes.

In vitro assays for studying kinesin-1 function

We have used two in vitro motility assays for these studies. Rat liver RER movement in the presence of *Xenopus* egg cytosol has been described previously (Allan and Vale, 1994), and we now demonstrate that this motility is driven by kinesin-1. This is in keeping with in vivo and in vitro observations of ER dynamics or distribution in different systems (Bannai et al., 2004; Feiguin et al., 1994; Lane and Allan, 1999).

The second motility assay uses a rat liver Golgi stack fraction combined with *Xenopus* egg cytosol, and under the conditions used here, this generates active vesicle movement. We find that both the DTC and uKHCct fusion proteins inhibit ~50% of this motility, identifying kinesin-1 as the motor for a substantial proportion of moving vesicles. The remaining vesicle translocation is driven by kinesin-2 (Wozniak & Allan, in preparation), with a minor contribution from as yet unidentified kinesin family members, which might include KIF1C (Dorner et al., 1998). Interestingly, the kinesin family member that drives the extension of membrane tubules in the Golgi fraction in the presence of brefeldin A is clearly distinct from the vesicle motors, since not only is tubule extension much slower than vesicle movement, it is also inhibited by the H1 monoclonal antibody (Robertson and Allan, 2000), whereas vesicle movement is not (data not shown).

The kinesin-1-positive vesicles in the Golgi fraction often contain ERGIC58, suggesting that they are involved in the Golgi-ERGIC-ER recycling pathway. The occasional presence of p115 would be consistent with this hypothesis. We saw no obvious overlap with other ERGIC components such as COPI or the KDEL receptor, however, suggesting that the vesicles were not fragments of the ERGIC: instead, they might be involved in traffic between the Golgi apparatus and late ERGIC, or between the ERGIC and the ER. This is in keeping with previous studies on the complexity and dynamics of the ERGIC (Marra et al., 2001). Larger vesicles were sometimes labelled with antibodies to FTCD, a Golgi protein that also cycles between the ERGIC and Golgi apparatus: while it partially overlaps with ERGIC58, p115 and COPI, its localisation is clearly distinct (Gao et al., 1998; Hennig et al., 1998).

Surprisingly, kinesin-1 was not present on vesicles containing secretory markers, even though kinesin-1 in other systems clearly transports vesicles between the TGN and plasma membrane (Wozniak et al., 2004). A small percentage of vesicles did possess both kinesin-1 and γ-adaptin or M6PR, suggesting that kinesin-1 may be involved in a subset of post-TGN traffic in rat liver. Taken together with the localisation of kinesin-1 to the Golgi region in vivo (this work; Gyoeva et al., 2000; Marks et al., 1994), these data support a role for kinesin-1 in traffic away from the Golgi apparatus back towards the ER, and for selected onwards traffic.

Function of specific KLC1 isoforms

It has been proposed, based on the use of antibodies which recognise limited sets of KLC1 isoforms (Gyoeva et al., 2000; Khodjakov et al., 1998) and on the molecular weight of KLCs present on particular cargo (Liao and Gundersen, 1998), that specific KLC isoforms target kinesin-1 to distinct structures. Here, we provide functional evidence in support of this model. We have made use of GST fusion proteins that contain the TPR domain and C-terminal

regions of KLC1B, D and KLC2, but which lack the N-terminus and KHC-binding heptad repeat domain. Strikingly, we found that only the KLC1B fusion protein inhibited RER movement, whilst KLC1D inhibited Golgi vesicle movement.

An antibody that recognizes both KLC1D and E has previously been shown to label the Golgi apparatus in cultured cells (Gyoeva et al., 2000). Since KLC1D is identical to KLC1E except for the inclusion of an additional 9 amino acids (fig. 1B), it is possible that both isoforms are present in the rat liver Golgi fraction, and that the KLC1D fusion protein inhibits both KLC1D and E function. Indeed, immunoblotting of the Golgi membranes revealed two KLC bands of similar molecular weight (fig. 1A). An interesting possibility is that these two isoforms correspond to two distinct kinesin-1-positive vesicle populations: those containing p58 and those positive for markers such as FTCD, M6PR or γ-adaptin.

Our functional data suggest that KLC1B is important for RER motility. In contrast, an antibody to this isoform labels mitochondria, rather than ER (Khodjakov et al., 1998). However, since KLC1B and C differ only by the insertion of 9 amino acids in the KLC1C c-terminus (fig. 1A), the anti-KLC1B antibody will also recognise KLC1C. It is possible that one of these splicing variants is specific for mitochondria, while the other is restricted to ER membranes, but this hypothesis remains to be tested.

Since the sole difference between the KLC1B and D constructs used here lies in the c-terminus, this demonstrates the importance of this region in cargo-specific functions of KLCs. It also suggests that the TPR domain, which interacts with many potential cargo proteins, has a more generic function in cargo binding. The only protein identified so far that interacts with the C-terminal region of a KLC is 14-3-3η, which has been reported to bind to phosphorylated KLC2 C-terminal (Ichimura et al., 2002). We are currently searching for KLC1B and D-interacting proteins in the RER and Golgi fractions.

A key question is how do the KLC1 fusion proteins lead to the inhibition of membrane-associated kinesin-1? If KLCs are needed for kinesin-1 to bind to cargo, then a simple possibility is that the appropriate fusion protein would compete for binding to a specific receptor on the membrane, releasing kinesin-1, and inhibiting movement. This would parallel the effects of the KLC_{ALL} antibody, which binds to the TPR domain and leads to loss of motor binding to axonal vesicles (Stenoien and Brady, 1997). This was not the case for KLC1 fusion proteins, however, as kinesin-1 remained membrane-bound although membrane motility was inactivated. This result could be explained if both KHCs and KLCs participate in cargo binding. Indeed, KHC dimers may be able to bind to membranous cargo in the absence of KLCs (Palacios and St Johnston, 2002; Skoufias et al., 1994), and fungal kinesin-1 lacks KLCs altogether (Seiler et al., 2000). Moreover, two potential kinesin-1 receptors have been identified on the ER—kinectin (Toyoshima et al., 1992) and the related p180 ribosome receptor (Diefenbach et al., 2004)—both of which interact with the KHC C-terminal domain (Diefenbach et al., 2004; Ong et al., 2000). If both heavy and light chains bind simultaneously to different cargo molecules, this could explain the tight membrane association that has been reported for kinesin-1 (Muresan et al., 1996; Tsai et al., 2000), and would be consistent with the undetectable level of kinesin-1 turnover on membranes that we have observed. Specific regulatory mechanisms would then be needed to control kinesin-1 cargo association, with one example being the phosphorylation of the C-terminal domain of KLC2 by glycogen synthase kinase 3β which enables Hsc70 to remove kinesin-1 from the membrane in neurons (Morfini et al., 2002).

Regulation of kinesin-1 activity

If release of kinesin-1 from the membrane is not the mechanism of inhibition, then what is? A likely alternative involves the ability of kinesin-1 to fold up so that the C-terminal domain of

KHC interacts with the neck region, so inhibiting ATPase activity (Coy et al., 1999; Friedman and Vale, 1999; Hackney and Stock, 2000; Stock et al., 1999) and the ability to move along microtubules (Coy et al., 1999). Although KHC dimers alone can fold in this way, the suppression of ATPase activity is even greater in the presence of KLCs (Coy et al., 1999; Friedman and Vale, 1999; Hackney and Stock, 2000; Stock et al., 1999), and it has been suggested that KLCs are vital to allow correct in vivo regulation of this folding process (Verhey et al., 1998). We propose that the appropriate KLC reagent (KLC1B for the RER, KLC1D for vesicles in the Golgi fraction) competes with endogenous KLC1 for binding to a cargo-specific receptor (protein X in fig. 9, I → II). Although kinesin-1 still remains membrane-bound by virtue of the uKHC-protein Y interaction, the release of KLC1 from protein X leads to a conformational change that exposes the critical region in the uKHCct, allowing kinesin-1 to fold up and therefore inhibiting motor activity (fig. 9, II).

When both heavy and light chain fusion proteins are combined, kinesin-1 is still not released from the membrane (fig. 5). This suggests that uKHCct is not able to compete for binding to protein Y (fig. 9). Instead, based on our results (figs 6 & 7) and those of others (Coy et al., 1999; Friedman and Vale, 1999; Hackney and Stock, 2000; Stock et al., 1999), we propose that uKHCct inhibits kinesin-1-driven membrane movement by binding to the neck region of the membrane-bound motor (fig. 9, III), thereby directly inhibiting its ability to hydrolyse ATP and move along microtubules. Interestingly, we find that this interaction leads to some kinesin-1 pelleting with microtubules in the presence of ATP (fig. 7), which fits with the observation that a short C-terminal fragment does not inhibit the binding of Drosophila kinesin-1 to microtubules, but instead actually increased the time spent associated with the microtubule three-fold (Coy et al., 1999). One possibility is that the interaction of exogenous C-terminus locks kinesin-1 in a different kinetic state compared to the ADP-bound form that binds only weakly to microtubules, and that is seen when the endogenous tail

interacts with the neck (Hackney and Stock, 2000). Alternatively, the association between native kinesin-1 and microtubules may be promoted by the ability of the C-terminal fragment to bind to microtubules directly (Coy et al., 1999; Hackney and Stock, 2000; fig. 7B). In full length KHC, this microtubule binding site is thought to remain hidden in the presence of light chains (Navone et al., 1992; Verhey et al., 1998), although it may be active in fungal kinesin-1, which lacks KLCs (Straube et al., 2006). We can rule out that uKHCct is inhibiting kinesin-1 activity simply by coating the entire microtubule surface, since it had no effect on the 50% of vesicle movement driven by kinesin-2.

The model we propose for the inactivation of kinesin-1 while it is membrane-bound is based on our observations following the addition of exogenous protein. It has previously been suggested that kinesin-1 is folded in the absence of cargo (i.e. when kinesin-1 is cytosolic), and that it becomes unfolded and activated following binding to its correct target (Coy et al., 1999; Friedman and Vale, 1999; Hackney and Stock, 2000; Stock et al., 1999; Verhey et al., 1998). However, since our data show that it is possible to inactivate membrane-associated kinesin-1, it is tempting to speculate that reversible folding may occur while kinesin-1 is attached to cargo, and that this may constitute a mechanism of regulation. Indeed, it is known that ER-associated kinesin-1 is inactive in Xenopus eggs and early embryos, but becomes activated when the membranes are incubated in cytosol prepared from somatic cells (Lane and Allan, 1999). What might trigger a regulated conformational change is not clear, but phosphorylation of the motor or an alteration in the conformation of protein X would be two possibilities. Indeed, if protein X were a transmembrane protein, this might provide a means of co-ordinating motor activity with the concentration of cargo in the vesicle or tubule.

In summary, we propose that KLCs must be correctly associated with cargo-specific molecules in order for kinesin-1 to be in the unfolded, active state (fig. 9). Given the KLC isoform-specific effects we observe, the variable C-terminal domain of KLCs is very likely to

be responsible for targeting kinesin-1 to particular cargoes. The existence of at least 19 splice forms of KLC1 (McCart et al., 2003) suggests that there are easily enough isoforms to account for the wide range of structures that this motor transports within the cell.

Materials and Methods

Additional Materials and Methods (antibodies and reagents; cloning and protein purification; immunofluorescence) are provided as supplementary material.

Preparation and manipulation of Xenopus extracts

Interphase-arrested Xenopus egg extracts were prepared from metaphase-arrested CSF extracts (Murray, 1991), or by preparation of extracts from laid eggs in the absence of EGTA (Newmeyer and Wilson, 1991). Cytosol fractions for motility assays were generated by centrifugation as described (Allan and Vale, 1991).

To immunodeplete kinesin-1 from *Xenopus* egg cytosol, protein G magnetic beads (Dynal) were washed with A/S buffer (100 mM K-Acetate, 3 mM Mg-Acetate, 5 mM EGTA, 10 mM HEPES, 150 mM sucrose, pH 7.4) and incubated with SUK4 or MYC antibodies for 1 hr at 4°C. The beads were washed with A/S buffer then 150 μl *Xenopus* cytosol was added and incubated for 1 hr at 4°C. To test the effect of GST-fusion proteins on *Xenopus* cytosol, 30 μg recombinant protein was bound to glutathione beads for 1 hr. Beads were washed twice with A/S buffer and incubated for 30 min at 4°C with 100 μl of interphase *Xenopus* cytosol. In both cases, cytosols were used immediately for motility assays.

Preparation of rat liver membrane fractions and motility assays

RER and stacked Golgi fractions were prepared from rat livers as described (Allan and Vale, 1994; Allan and Vale, 1991). Membranes were incubated for 10 – 13 min on ice with the indicated recombinant proteins before assaying motility. 0.5 – 0.8 μl RER (4.5-7.2 μg) or 1μl Golgi fraction membranes (2.7 μg) were added to 9 μl of *Xenopus* cytosol and incubated in a microscope flow cell in a humid chamber (Allan and Vale, 1991) that had been pre-

coated with microtubules. Taxol-stabilised porcine brain tubulin microtubules (0.2 mg/ml in BRB80: 80 mM K-PIPES, 2 mM $MgCl_2$, 1 mM EGTA pH 6.8) were incubated in the flow cell for 20 – 30 min and washed 3 times with A/S buffer with energy mix before flowing in the cytosol-membrane mixture. For supplementary fig. 1A and B, cytosol was replaced by A/S buffer with energy mix. Golgi fraction motility assays were incubated for 10 min and then 20 random fields were observed for 30 sec each by VE-DIC microscopy (Lane and Allan, 1999). The average number of moving vesicles per field was determined. RER motility assays were incubated for 60 min in the flow cell to allow ER network formation and then 30 random fields were recorded, and the number of three-way junctions in the ER network was counted (Allan, 1995).

Biochemical analysis of membranes

To test if *Xenopus* egg kinesin-1 was recruited onto rat liver membranes, 10 μl of membranes were incubated with 50 μl of *Xenopus* cytosol for 10 – 20 min at RT in the presence of nocodazole, and then pelleted through a 0.8 M sucrose cushion in a Beckman TLA-100 rotor at 55,000 rpm, 4°C for 30 min. To test the influence of fusion proteins on kinesin-1 membrane association, 10 μl of membranes were incubated with 20 μl of fusion protein for 30 min on ice and centrifuged as above. The pellets were washed with A/S buffer analysed by immunoblotting.

To test whether GST-fusion proteins affect kinesin-1 conformation, 80 μg Golgi membranes were incubated with 20 μg recombinant proteins for 10 min on ice, then lysed for 10 min on ice by the addition of an equal volume of 2% Triton TX-100, 400 mM NaCl, 100 mM ascorbic acid and protease inhibitors in acetate buffer. After centrifugation at full speed in a microfuge for 10 min at 4°C, the lysates were loaded onto 650 μl 5 – 20 % sucrose gradients (in acetate buffer), centrifuged in a Beckman SW55 rotor with adapters at 4°C,

46,600 rpm for 5 hr. Fractions were collected from the bottom of the tubes and analysed by immunoblotting.

Microtubule binding assays

RER membranes (400 μg) or interphase *Xenopus* cytosol (200 μg) were incubated for 15 min at RT with 200 μl of A/S buffer or A/S buffer containing 0.3 mg/ml GST-fusion proteins. Samples were lysed for 10 min on ice after the addition of an equal volume of 2 % Triton TX-100, 400 mM NaCl plus protease inhibitors and centrifuged for 10 min at 4°C, full speed in a microfuge. Cleared lysates were supplemented with either 5 mM ATP, 20 μM taxol, 1mM DTT, 1 μg/ml cytochalasin D (lysates preincubated with recombinant proteins) or 10 U/ml hexokinase, 20 μM glucose, 20 μM taxol, 400 μM AMP-PNP, 1mM DTT (lysates preincubated with buffer) and left at RT for 5 min. Microtubules (50 μg) were added and incubated for 30 min at 30ºC. Samples were centrifuged for 30 min at 32,000 rpm, 4°C in Beckman TLS55 rotor through a 40 % (w/v) sucrose cushion in BRB80 buffer supplemented with 1 mM DTT, 4 μM taxol, 1 μg/ml cytochalasin D and protease inhibitors. Pellets were washed in BRB80 buffer with 1 mM DTT, 4 μM taxol, 1 μg/ml cytochalasin D and protease inhibitors and spun as above for 20 min. Pellets were resuspended in 15 μl BRB80 and analysed by immunoblotting.

GST pull-downs from COS7 cell lysates

KIF5B$_{NT}$-GFP (supplemental methods) was expressed in COS7 cells after transfection using JetPei (Polyplus-transfection, France). Cells were washed once with PBS, lysed in lysis buffer (10 % glycerol, 1 % Triton TX-100, 1.5 mM $MgCl_2$, 50 mM HEPES, pH 7.5, 150 mM NaCl, 1 mM EGTA, 50 mM ascorbic acid) and spun for 10 min in a microfuge at full speed, 4°C. Recombinant kinesin-1 GST-fusion proteins (5 μg each) were immobilised on

glutathione beads for 1 hr, then washed twice with acetate buffer, 1 % Triton TX-100, 150 mM NaCl. Beads were then incubated with 100 μg of COS7/KIF5B_{NT}-GFP lysate in a total volume 100 μl for 2 hr at 4°C. Beads were washed three times as above and mixed with SDS-PAGE loading buffer.

Supplementary information is available at *The EMBO Journal* Online.

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Figure legends

Figure 1. Different KLC isoforms are present on the Golgi and RER membranes

(**A**) Alignment of variable C-terminal regions of KLC1 splicing variants. Amino acid numbers are given. (**B**) Rat liver membranes were analysed by immunoblotting with KLC_{ALL} and uKHC antibodies. (**C**) Schematic representation of kinesin-1 structure and the GST-fusion proteins used in this study. (**D**) SDS-PAGE analysis of purified recombinant proteins were separated by SDS-PAGE. The position of molecular weight markers is indicated.

Figure 2. Kinesin-1 fusion proteins inhibit motility in the RER fraction

RER membranes were incubated with BTC or DTC (**A**); or 2TC (**B**); or uKHCct (**C**) and effects on membrane movement in the presence of cytosol was analysed. GST was used as a control. The extent of RER network formation was analysed by counting the number of three way junctions, and an average (± standard error) is shown. T tests were performed and P-values are given, with asterisks indicating which values were compared.

Figure 3. Golgi fraction vesicle movement is inhibited by KLC1D but not KLC1B

1 μl of Golgi membranes was incubated with indicated amount of GST, BTC or DTC (**A**); or uKHCct alone or together with BTC, DTC or 2TC (**B**). Vesicle motility in the presence of cytosol was analysed. The bars show average number (± S.E.M.) of moving vesicles during 10 min (20 fields, 30 sec each). T tests were performed and P- values are shown, with asterisks indicating which values were compared.

Figure 4. Membrane motility does not require cytosolic kinesin-1

(**A**) 10 μl Golgi and RER membranes were incubated with 50 μl of *Xenopus* cytosol for 10 – 20 min at RT, collected by centrifugation through a sucrose cushion, and then uKHC was detected by immunoblotting. Untreated *Xenopus* cytosol and Golgi and RER membranes were loaded as controls. (**B**) *Xenopus* cytosol was depleted of kinesin-1 using SUK4 antibodies, with MYC antibodies used as a control. The immunodepleted cytosols and untreated samples were immunoblotted with uKHC antibody. To demonstrate equal loading, the Ponceau-S stained membrane is shown. (**C**) Motility assays were performed using depleted *Xenopus* egg cytosols and results are shown as the average number of three way junctions (RER, left), or moving vesicles (Golgi fraction, right). SUK4 depleted cytosols were used to test the effect of GST-BTC, GST-BTC and GST-uKHCct on the motility of RER (**D**) and Golgi (**E**) membranes. The error bars represent standard error, and P-values from T tests are shown.

Figure 5. Recombinant proteins do not release kinesin-1 from membranes

10 μl of either RER (**A**) or Golgi membranes (**B**) were incubated for 30 min on ice with 20 μl GST-fusion proteins, centrifuged through a sucrose cushion and analysed by immunoblotting with uKHC and KLC_{ALL} antibodies.

Figure 6. GST-KHCct binds to the motor domain of kinesin-1

(**A**) GFP tagged amino-terminus of kinesin-1 (schematically represented below gels) was expressed in COS7 cells. Cells lysates were incubated with the indicated GST fusion proteins bound to glutathione beads. Proteins bound to the beads were separated by SDS-PAGE and transferred onto nitrocellulose membrane, which was then stained with Ponceau S (lower panel) and tested with anti-GFP (upper panel). (**B**) 80 μg of Golgi membranes were pre-

incubated with 20 μg of the indicated GST fusion protein, solubilised and separated on 5–20% sucrose gradients by ultracentrifugation. uKHC was detected by immunoblotting.

Figure 7. GST-KHCct induces rigor binding of kinesin-1 to microtubules

RER membranes (**A**) and *Xenopus* egg cytosol (**B**) were incubated with GST-fusion proteins, lysed and cleared by centrifugation. Lysates were supplemented with either 5 mM ATP (samples containing GST-fusion proteins) or 400 μM AMP-PNP and incubated with microtubules for 30 min. For the control, no microtubules were added. Microtubules were pelleted through a 40 % sucrose cushion. Pellets were separated by SDS-PAGE and proteins were transferred onto nitrocellulose, stained with Ponceau and tested for the presence of kinesin-1 (uKHC antibody) and kinesin-2 (K2.4 antibody).

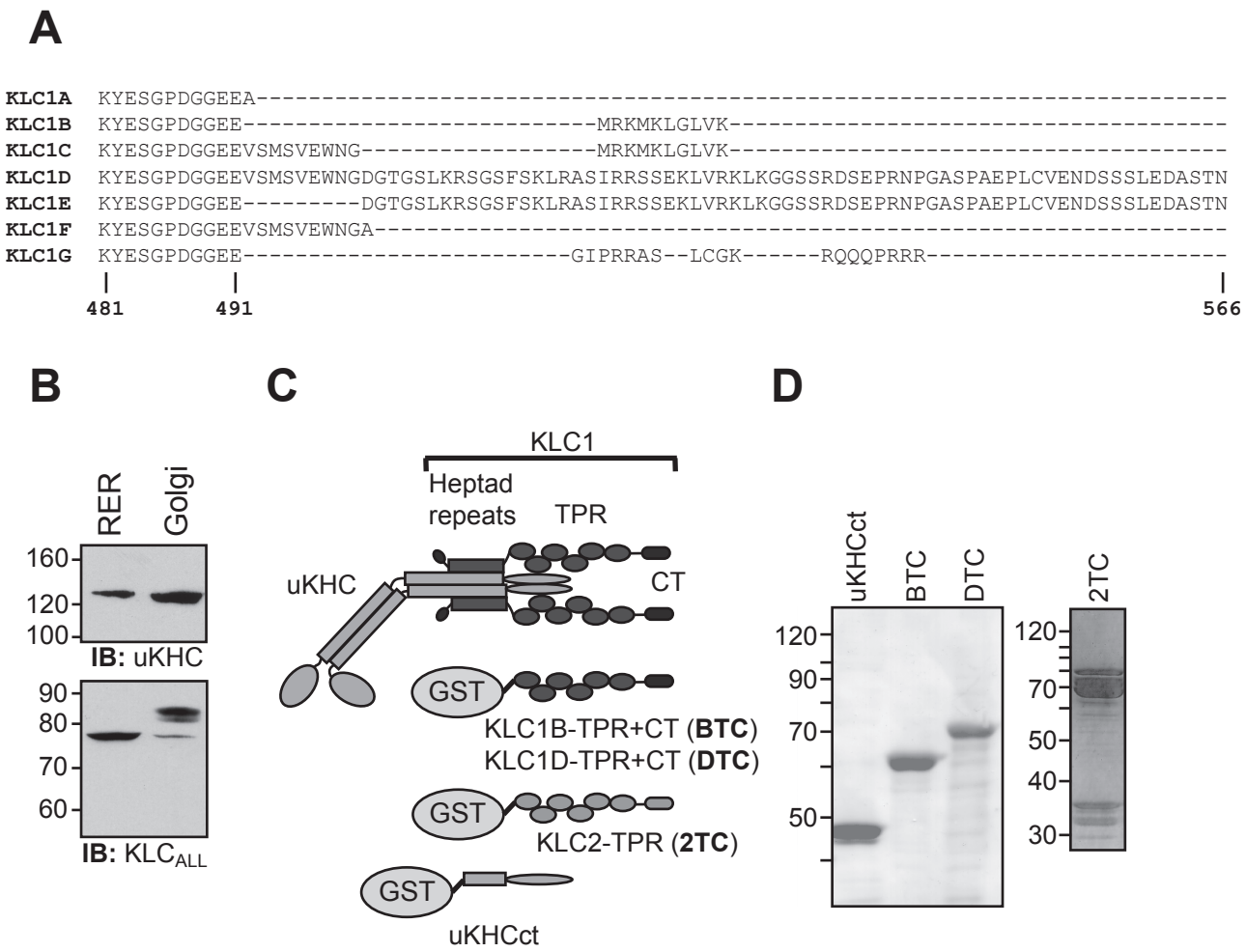
Figure 8. Identification of vesicular cargoes of kinesin-1

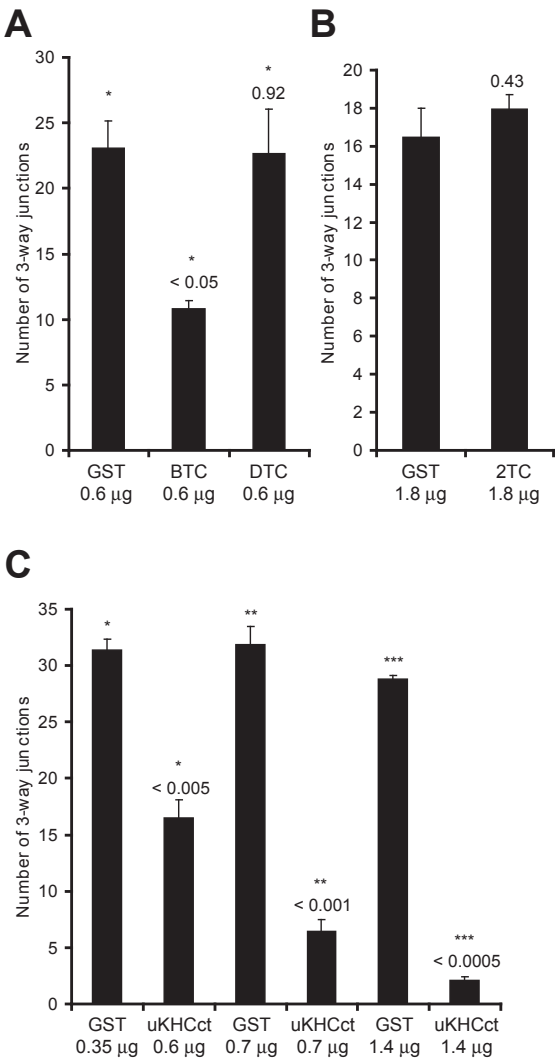
Golgi membranes were labelled with H2 (**A, B, E**) or HD (**C, D**) anti-kinesin-1 antibodies in parallel with the ERGIC marker anti-p58 (**A**), the Golgi apparatus markers anti-p115 (**B**) and anti-FTCD/58k (formiminotransferase cyclodeaminase; **C**), and the TGN markers anti-γ-adaptin (**D**) and anti-M6PR (**E**). Arrows indicate colocalisation and arrowheads its absence. Scale bar = 2 μm.

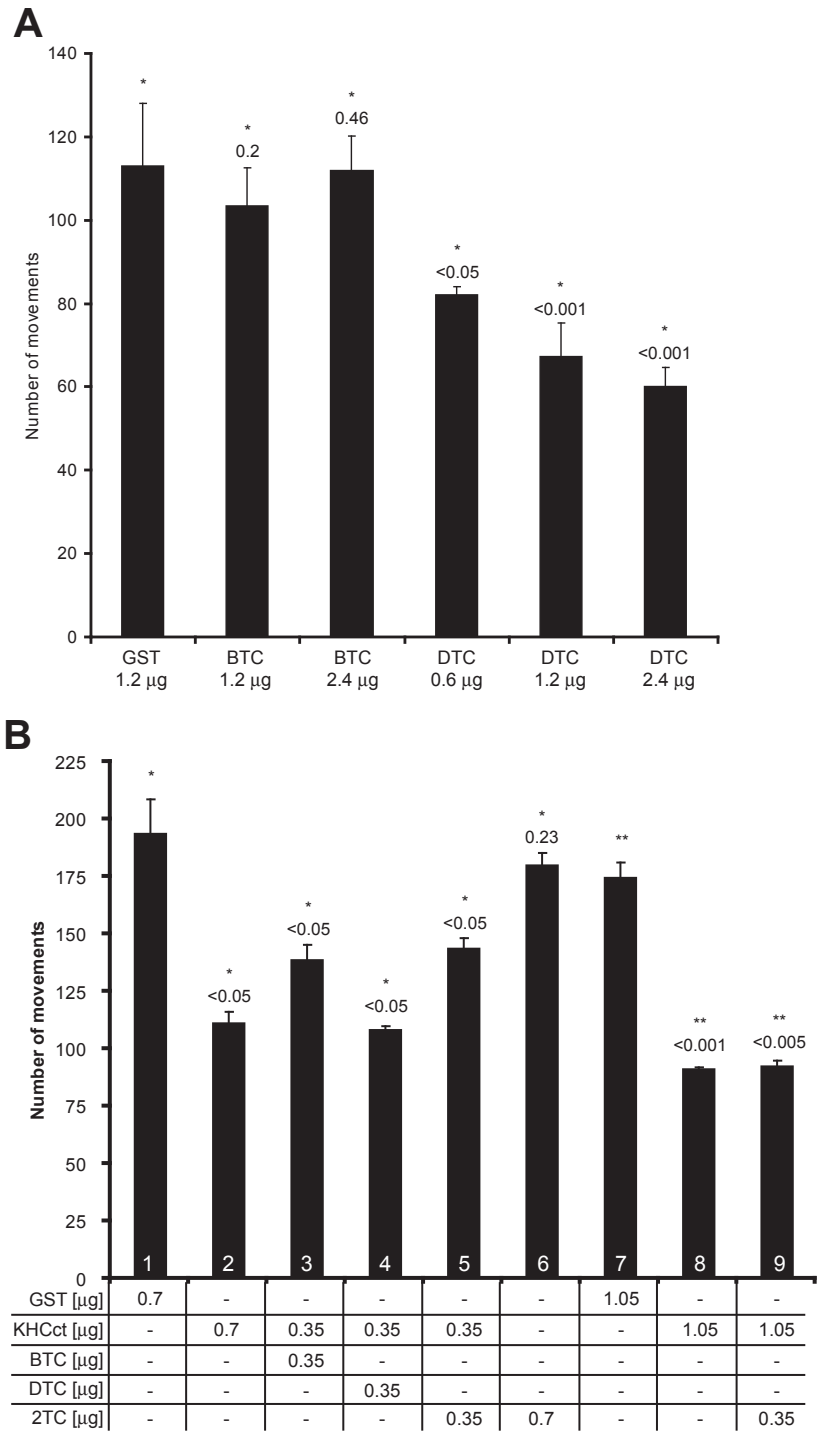
Figure 9. Model for kinesin-1 attachment to membranes and the effect of recombinant proteins

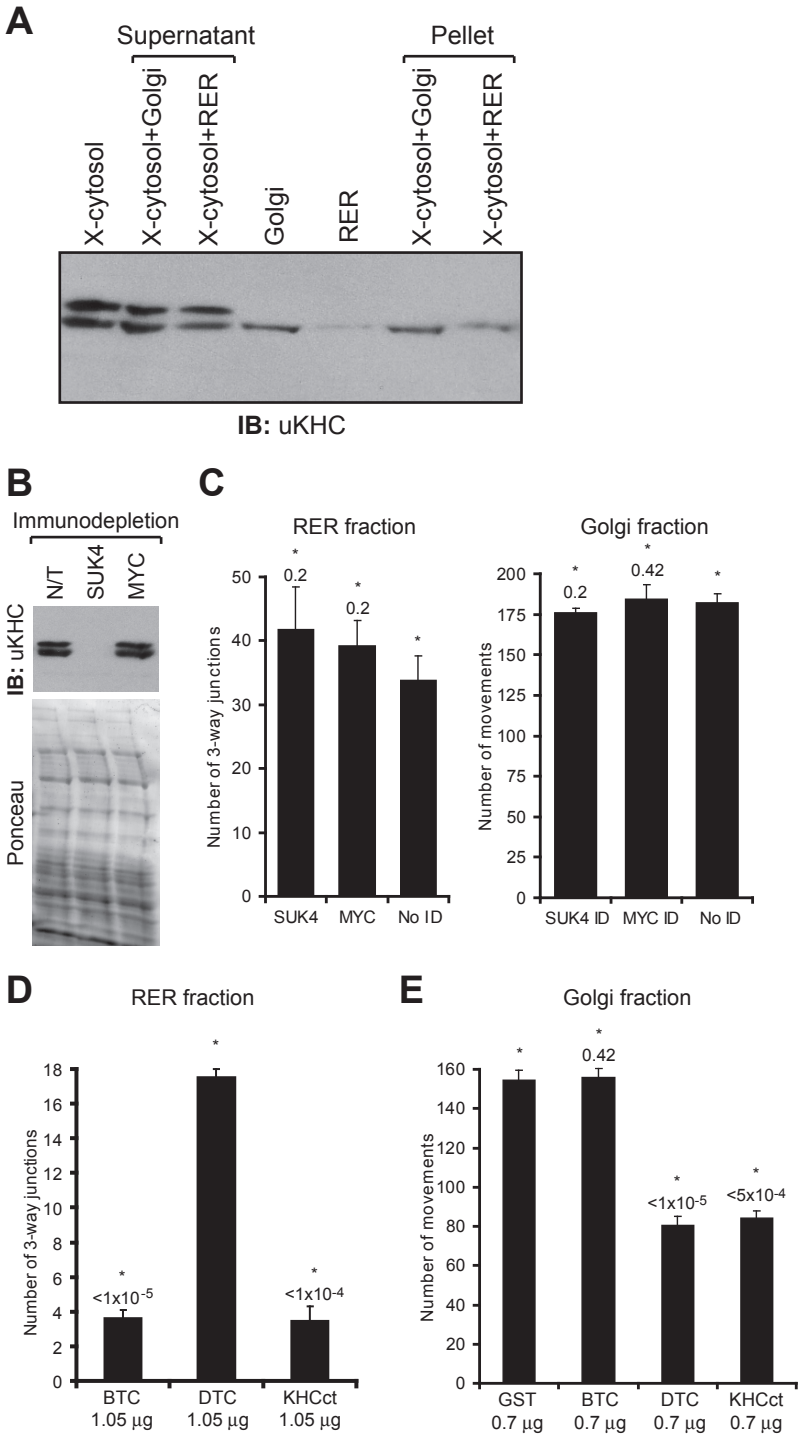
Kinesin-1 is anchored to membranes via KHC and KLC, and is active. The KLC anchor determines the specificity and targeting to membranes via interaction with protein X, which will be different on Golgi and RER (**I**). The addition of KLC1 fusion protein releases KLCs and allows stabilisation of the folded, inactive conformation (**II,** Verhey *et al.*, 1998). The

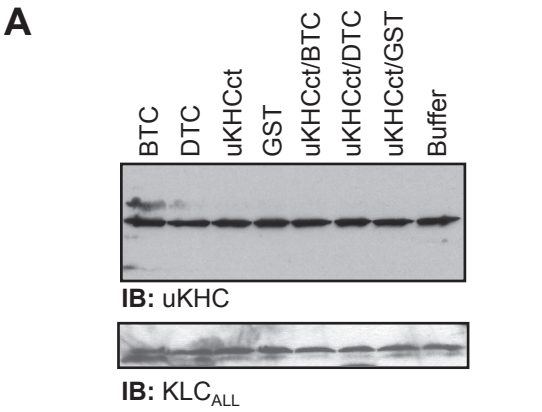
addition of the uKHCct fragment does not interfere with cargo binding, but inhibits the motor domain by triggering rigor binding to the microtubule (**III**). The addition of both uKHC and KLC reagents still does not release kinesin from membranes because the interaction with protein Y is retained and the recombinant KHCct and KLC1 constructs do not interact with each other. (**IV**). The GST fusion proteins are depicted as monomers for simplicity, but they may exist as dimers.

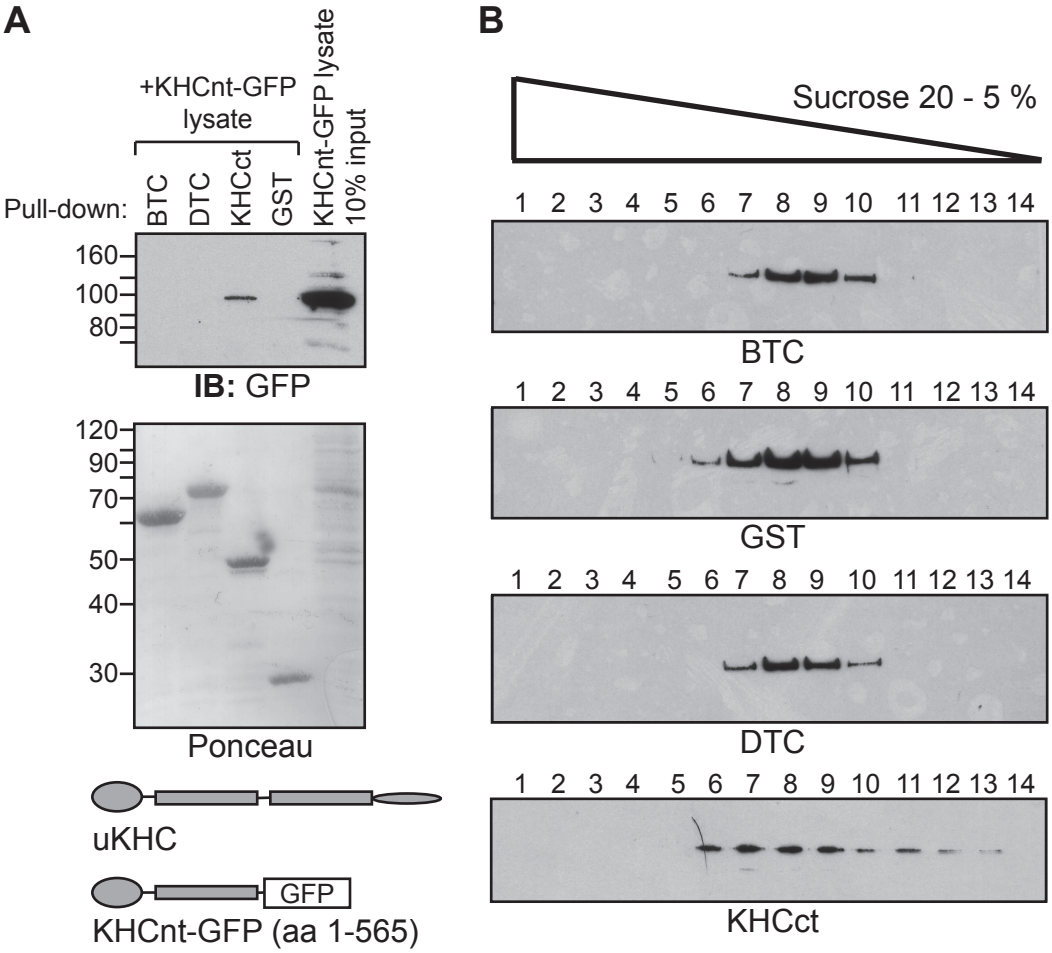

A
KLC1A KYESGPDGGEEA----------
KLC1B KYESGPDGGEE----------MRKMKLGLVK----------
KLC1C KYESGPDGGEEVSMSVEWNG----------MRKMKLGLVK----------
KLC1D KYESGPDGGEEVSMSVEWNGDGTGSLKRSGSFSKLRASIRRSSEKLVRKLKGGSSRDSEPRNPGASPAEPLCVENDSSSLEDASTN
KLC1E KYESGPDGGEE---------DGTGSLKRSGSFSKLRASIRRSSEKLVRKLKGGSSRDSEPRNPGASPAEPLCVENDSSSLEDASTN
KLC1F KYESGPDGGEEVSMSVEWNGA----------
KLC1G KYESGPDGGEE----------GIPRRAS--LCGK------RQQQPRRR----------
481
491
566
B
RER
Golgi
160
120
100
IB: uKHC
90
80
70
60
IB: KLC$_{ALL}$
C
KLC1
Heptad repeats
TPR
uKHC
CT
GST
KLC1B-TPR+CT (**BTC**)
KLC1D-TPR+CT (**DTC**)
GST
KLC2-TPR (**2TC**)
GST
uKHCct
D
uKHCct
BTC
DTC
2TC
120
90
70
50
120
70
50
40
30

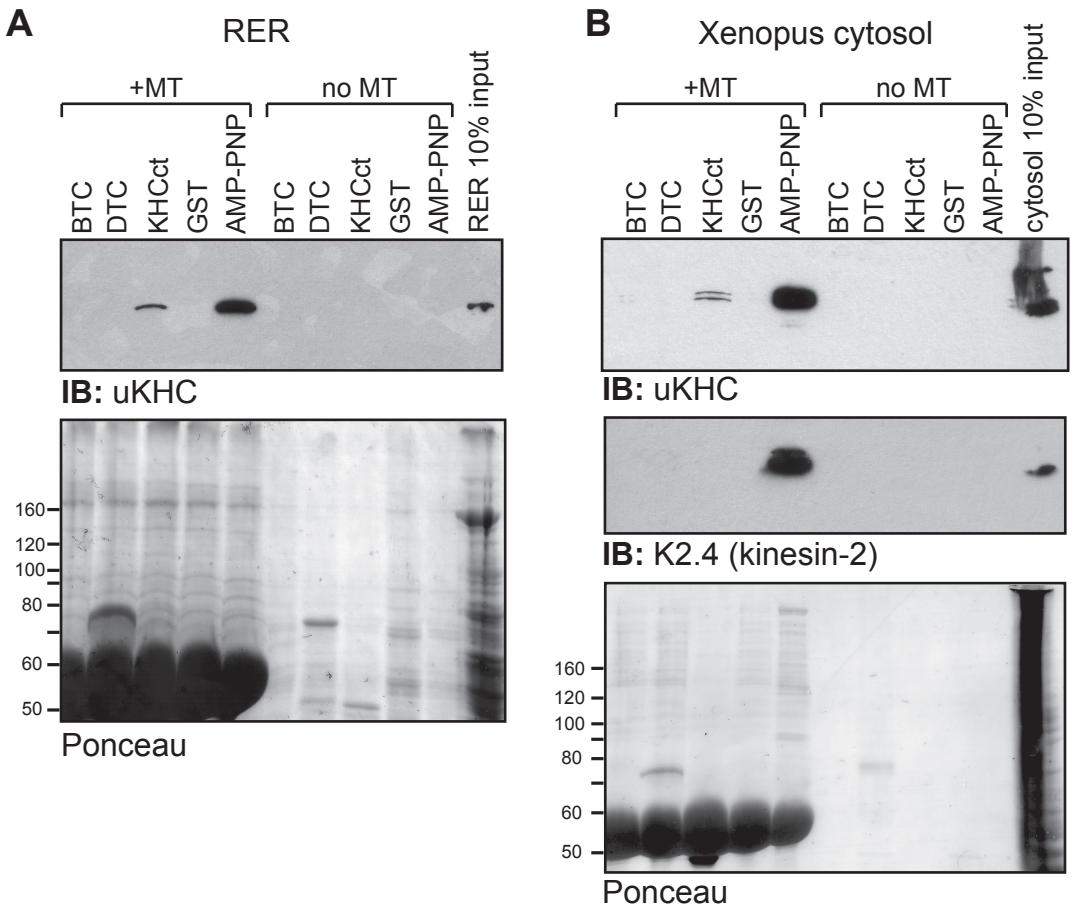

A
B
C
Number of 3-way junctions
GST 0.6 µg
BTC 0.6 µg
DTC 0.6 µg
0.92
< 0.05
GST 1.8 µg
2TC 1.8 µg
0.43
uKHCct 0.6 µg
GST 0.7 µg
uKHCct 0.7 µg
GST 1.4 µg
uKHCct 1.4 µg
< 0.005
< 0.001
< 0.0005

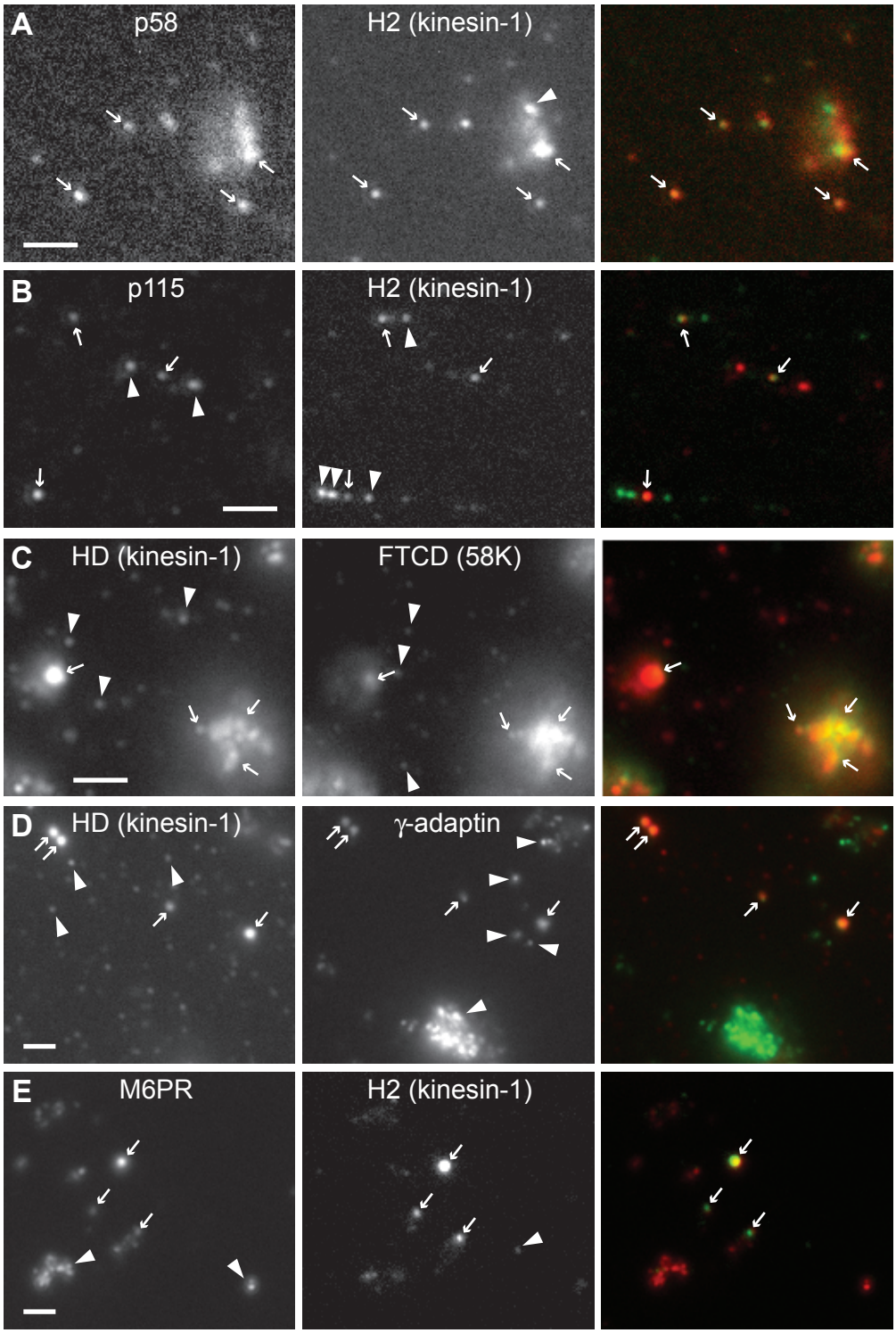

A
Number of movements
140
120
100
80
60
40
20
0
*
*
0.2
*
0.46
*
<0.05
*
<0.001
*
<0.001
GST 1.2 µg
BTC 1.2 µg
BTC 2.4 µg
DTC 0.6 µg
DTC 1.2 µg
DTC 2.4 µg
B
Number of movements
225
200
175
150
125
100
75
50
25
0
*
*
<0.05
*
<0.05
*
<0.05
*
<0.05
*
0.23
**
**
<0.001
**
<0.005
1
2
3
4
5
6
7
8
9
GST [µg] 0.7 - - - - - 1.05 - -
KHCct [µg] - 0.7 0.35 0.35 0.35 - - 1.05 1.05
BTC [µg] - - 0.35 - - - - - -
DTC [µg] - - - 0.35 - - - - -
2TC [µg] - - - - 0.35 0.7 - - 0.35

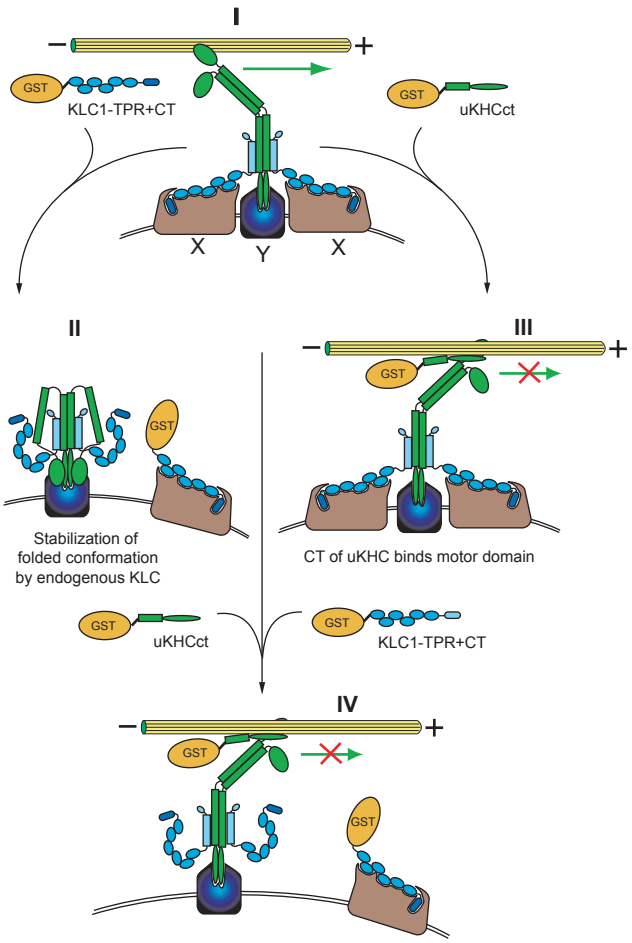

A
Supernatant
Pellet
X-cytosol
X-cytosol+Golgi
X-cytosol+RER
Golgi
RER
X-cytosol+Golgi
X-cytosol+RER
IB: uKHC
B
Immunodepletion
N/T
SUK4
MYC
IB: uKHC
Ponceau
C
RER fraction
Golgi fraction
Number of 3-way junctions
Number of movements
0.2
0.2
0.2
0.42
SUK4
MYC
No ID
SUK4 ID
MYC ID
No ID
D
RER fraction
Number of 3-way junctions
<1x10-5
<1x10-4
BTC 1.05 µg
DTC 1.05 µg
KHCct 1.05 µg
E
Golgi fraction
Number of movements
0.42
<1x10-5
<5x10-4
GST 0.7 µg
BTC 0.7 µg
DTC 0.7 µg
KHCct 0.7 µg


A
BTC
DTC
uKHCct
GST
uKHCct/BTC
uKHCct/DTC
uKHCct/GST
Buffer
IB: uKHC
IB: KLC$_{ALL}$
B
BTC
DTC
uKHCct
Buffer
GST
GST
uKHCct/BTC
uKHCct/DTC
uKHCct/GST
IB: uKHC
IB: KLC$_{ALL}$


A
+KHCnt-GFP lysate
KHCnt-GFP lysate 10% input
Pull-down:
BTC
DTC
KHCct
GST
160
100
80
IB: GFP
120
90
70
50
40
30
Ponceau
uKHC
GFP
KHCnt-GFP (aa 1-565)
B
Sucrose 20 - 5 %
1 2 3 4 5 6 7 8 9 10 11 12 13 14
BTC
GST
DTC
KHCct


A
RER
+MT
no MT
BTC
DTC
KHCct
GST
AMP-PNP
BTC
DTC
KHCct
GST
AMP-PNP
RER 10% input
IB: uKHC
160
120
100
80
60
50
Ponceau
B
Xenopus cytosol
+MT
no MT
BTC
DTC
KHCct
GST
AMP-PNP
BTC
DTC
KHCct
GST
AMP-PNP
cytosol 10% input
IB: uKHC
IB: K2.4 (kinesin-2)
160
120
100
80
60
50
Ponceau


A
p58
H2 (kinesin-1)
B
p115
H2 (kinesin-1)
C
HD (kinesin-1)
FTCD (58K)
D
HD (kinesin-1)
γ-adaptin
E
M6PR
H2 (kinesin-1)


I
GST
KLC1-TPR+CT
GST
uKHCct
X
Y
X
II
GST
Stabilization of
folded conformation
by endogenous KLC
III
GST
CT of uKHC binds motor domain
GST
uKHCct
GST
KLC1-TPR+CT
IV
GST
GST

Supplementary Materials and Methods

Antibodies and reagents

Antibodies were generously provided by following people: SUK 4 hybridoma (Ingold et al., 1988) from Dr J. Scholey (University of California, Davis, CA); KLC_{ALL} (Stenoien and Brady, 1997) from Dr S. Brady (University of Illinois, Chicago, IL); rabbit KLC (Khodjakov et al., 1998), from Dr F. Gyoeva (Russian Academy of Sciences, Moscow, Russia); rabbit anti-uKHC (Niclas et al., 1994) from Dr R. Vale (University of California, San Francisco, CA); HD anti-pankinesin antibody (Gyoeva and Gelfand, 1991) from Dr V. Gelfand (Northwestern University, Chicago, IL); rabbit anti-TGN38 (Luzio et al., 1990) and anti-M6PR (Reaves et al., 1996) from Dr P. Luzio (University of Cambridge, UK); anti-βCOP CM1A10 (Palmer et al., 1993) from Dr R. Pepperkok (E.M.B.L., Heidelberg, Germany); rabbit anti-p58 (Saraste and Svensson, 1991) from Dr J. Saraste (University of Bergen, Norway); sheep anti-Golgin 84 (Diao et al., 2003) from Dr M. Lowe (University of Manchester, UK). Polyclonal sheep antibody against GST was affinity purified as described (Addinall et al., 2001). The following monoclonal antibodies were purchased: H2 anti-KHC, Chemicon International (Temecula, CA); anti-apoB, Fitzgerald Industries International (Concord, MA); anti-γ-adaptin and anti-GM130, Transduction Laboratories (Lexington, KY); anti-KDELR, Calbiochem (EMD Biosciences, Darmstadt, Germany), anti-transferrin receptor (clone OX-26), Abcam (Cambridge, UK); anti-FTCD (clone 58K-9), Sigma (Saint Louis, MO). Sheep anti-rat albumin was from Bethyl Laboratories (Montgomery, TX), and rabbit anti-sec23 was from Affinity Bioreagents (Golden, CO).

Cloning and protein purification

The cDNA fragments encoding aa 228-543 of hamster KLC1B, 228-605 of hamster KLC1D (Khodjakov et al., 1998), both provided by Dr F. Gyoeva, 155-599 of mouse KLC2 (Rietdorf et al., 2001) provided by Dr M. Way (CRUK London Research Institute, London) and 771-

963 of rat uKHCct were amplified using specific primers and cloned into the pGEX bacterial expression vector. Protein production was induced for 2 hr with 0.1 mM IPTG in BL21 bacteria, which were subsequently lysed in PBS, 0.5 mg/ml lysozyme, 10 mM DTT, 1 mM PMSF, 1 % TX-100 and disrupted by sonication. The lysates were cleared by centrifugation at 13,000 rpm in a Beckman JA 25.50 rotor and GST-fusion proteins were purified on GSTrap FF columns (Amersham Biosciences) on a Dionex FPLC system. The eluted proteins were dialysed into acetate sucrose buffer (A/S: 100 mM K-Acetate, 3 mM Mg-Acetate, 5 mM EGTA, 10 mM HEPES, 150 mM sucrose, pH 7.4) and the final concentrations were 0.7 mg/ml (14.3 μM uKHCct) or 1.2 mg/ml for KLC proteins (18.9 μM – BTC; 17.1 μM – DTC; 15.7 μM – 2TC). When the effects of uKHC and KLCs were compared within the same experiment, KLC protein stocks were diluted to 0.7 mg/ml.

The cDNA encoding the amino-terminus of kinesin heavy chain (aa 1-565) was amplified with specific primers and cloned into the pEGFP vector (Clontech), generating $KIF5B_{NT}$-GFP.

Immunofluorescence

The immunofluorescence was performed according to Bananis and co-workers (Bananis et al., 2003). 1 μl of membranes was mixed with 9 μl ABC buffer (A/S buffer, 4 mM DTT, 20 μM taxol, 2 mg/ml BSA, 5 mg/ml casein, 400 μM AMP-PNP) and the mixture was flowed into a microtubule-coated flow cell. After a 20-30 minute incubation at RT, unbound membranes were washed away with 40 μl ABC buffer. The membranes were then incubated with 20 μl of diluted antibodies and incubated at RT for 6 min. Unbound antibodies were washed away with 40 μl ABC buffer and the membranes were incubated with diluted fluorophore-conjugated secondary antibodies in ABC buffer. The flow cell was then washed with 120 μl ABC buffer and observed using an Olympus BX60 upright microscope equipped with a CoolSnap ES camera (Roper Scientific) driven by Metamorph software (Universal

Imaging Corp.). When antibodies recognising lumenal components (apoB, albumin) were used, samples were prepared, fixed and permeabilised as described (Allan and Vale, 1994). For immunofluorescence analysis of NRK cells, cells were grown on 13 mm coverslips, fixed in methanol at -20°C for 5 min, and then labelled with primary and fluorescently-conjugated secondary antibodies and imaged as above.

Supplementary references

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Supplementary Figure legends

Supplementary figure 1. Effects of cytosol and recombinant proteins on RER and Golgi membrane movement

RER (**A**) and Golgi (**B**) membranes were mixed with *Xenopus* egg cytosol or A/S buffer plus energy mix and motility was analysed, demonstrating that motility requires cytosol. The effects of kinesin-1 fusion proteins (BTC, DTC and uKHCct, with GST as a control) on RER network formation (C) and Golgi vesicle attachment to microtubules (D) are demonstrated by representative fields from motility assays (see Materials and Methods). Scale bar = 10 μm. *Xenopus* egg cytosol (100 μl) was pre-incubated for 30 min with 30 μg of each recombinant protein bound to glutathione beads, recovered, and used for motility assays with either 0.5 μl RER (**E**) or 1 μl Golgi (**F**) membrane fraction. For panels A, B, E and F, the error bars represent standard error of three repeats, and P-values from T tests are shown. All data were compared to the GST control.

Supplementary Figure 2. Kinesin-1 antibodies colocalise with each other

Golgi membranes were labelled with mouse H2 and rabbit HD anti-KHC antibodies (**A**, upper panel), or H2 and rabbit KLC (**A**, lower panel) antibodies to demonstrate that they recognise the same structures, whereas distinct structures were labelled by H2 and Golgin 84 (**B**), or H2 and KDELR (**C**) antibodies. Scale bar = 2 μm.

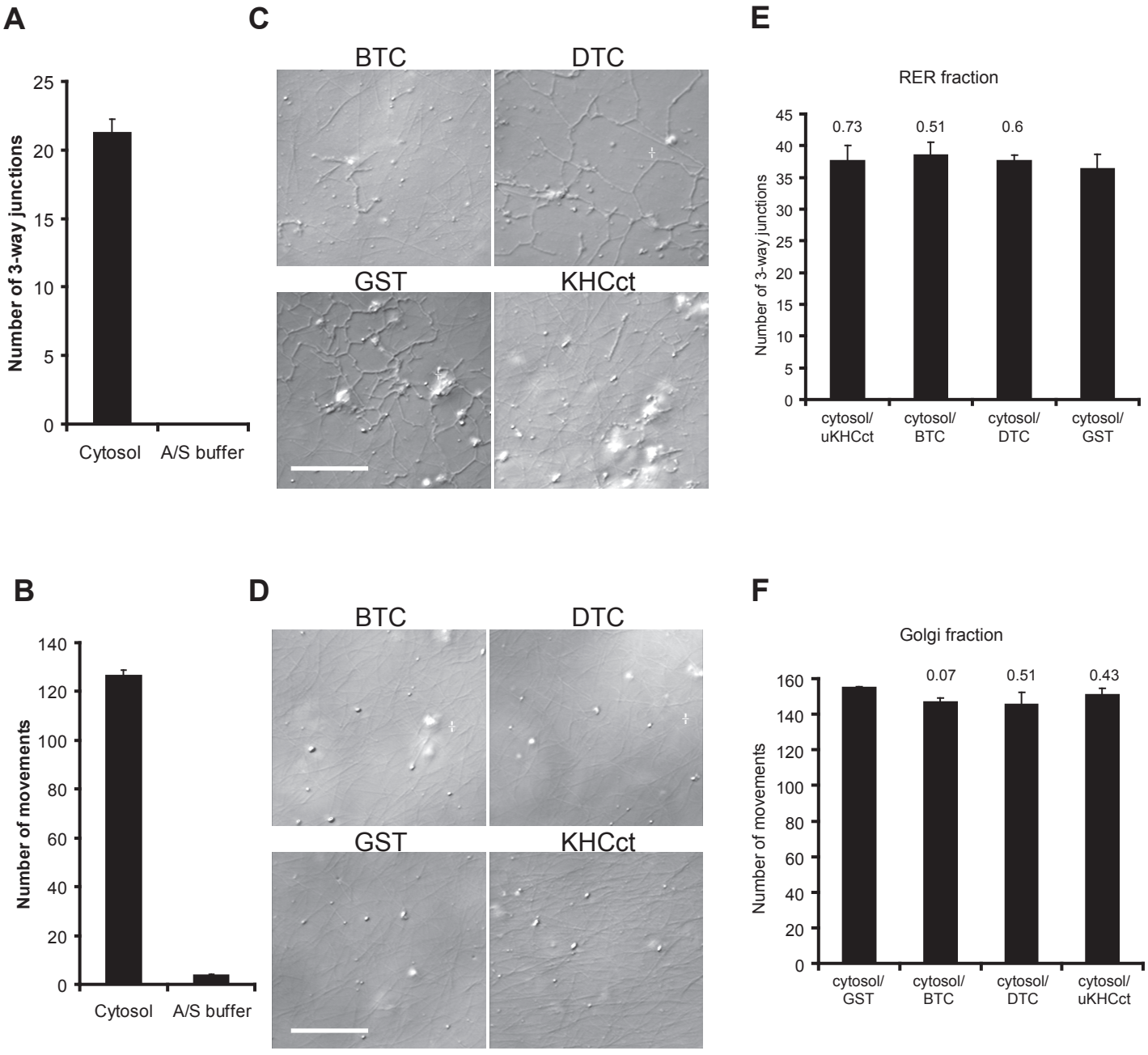
Supplementary Figure 3. Kinesin-1 antibodies partially colocalise with the ERGIC marker p58 and γ-adaptin in NRK cells

NRK cells fixed in methanol were labelled with H2 (**A, B**) and HD (**A, C**) anti-KHC antibodies together with antibodies to p58/ERGIC58 (**B**), an ERGIC marker, and γ-adaptin (**C**). Scale bar = 20 μm.

Supplementary Figure 4. Kinesin-1 colocalises with the Golgi-associated protein FTCD

NRK cells fixed in methanol were labelled with the HD anti-kinesin antibody in combination with FTCD/58k (**A, B**) and GM130 (**C**) antibodies which label the Golgi apparatus. The insert in **A** is a 2.25 magnification of the selected region. Scale bars are 10 μm.

Supplementary figure 1



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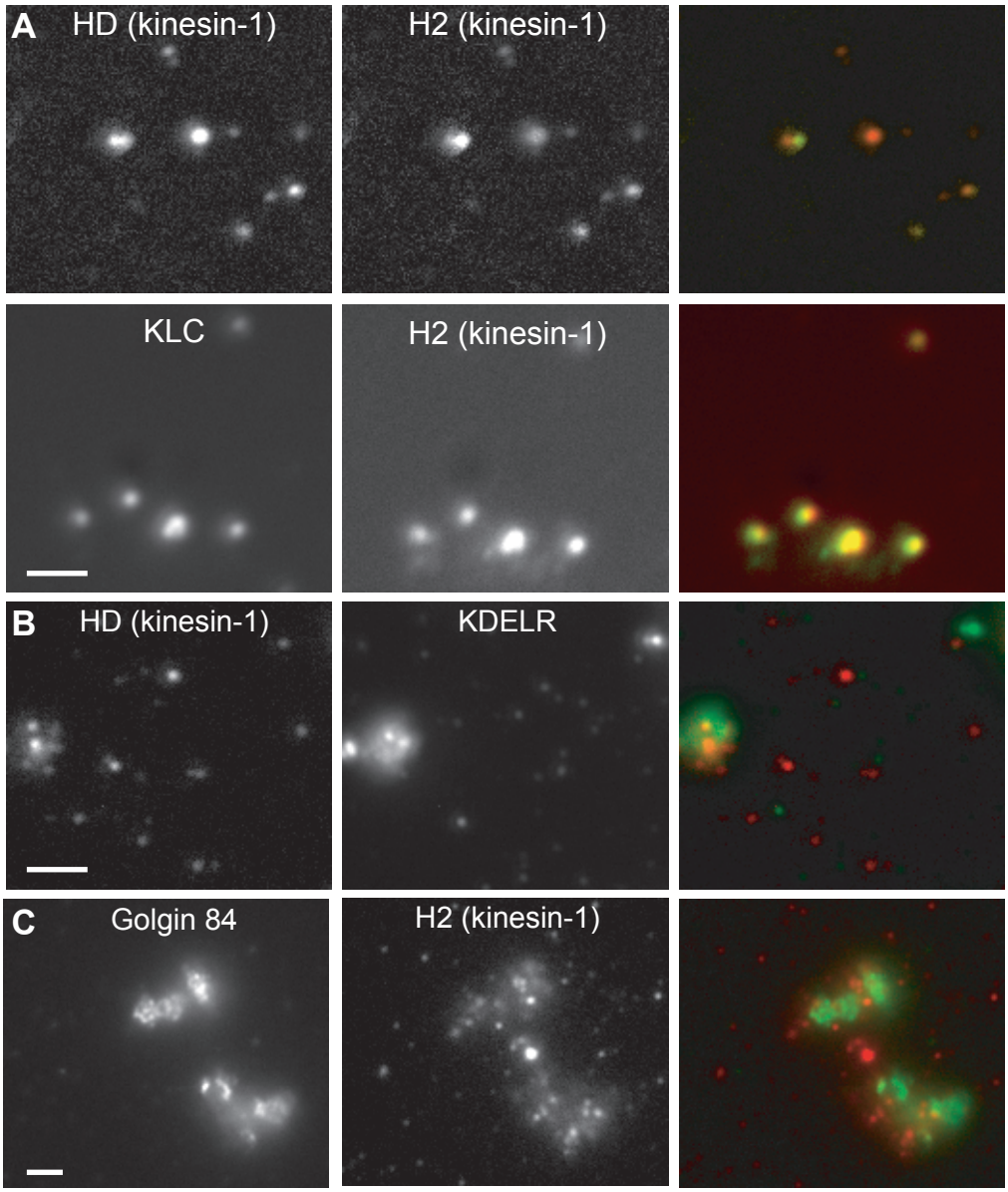
Supplementary figure 2



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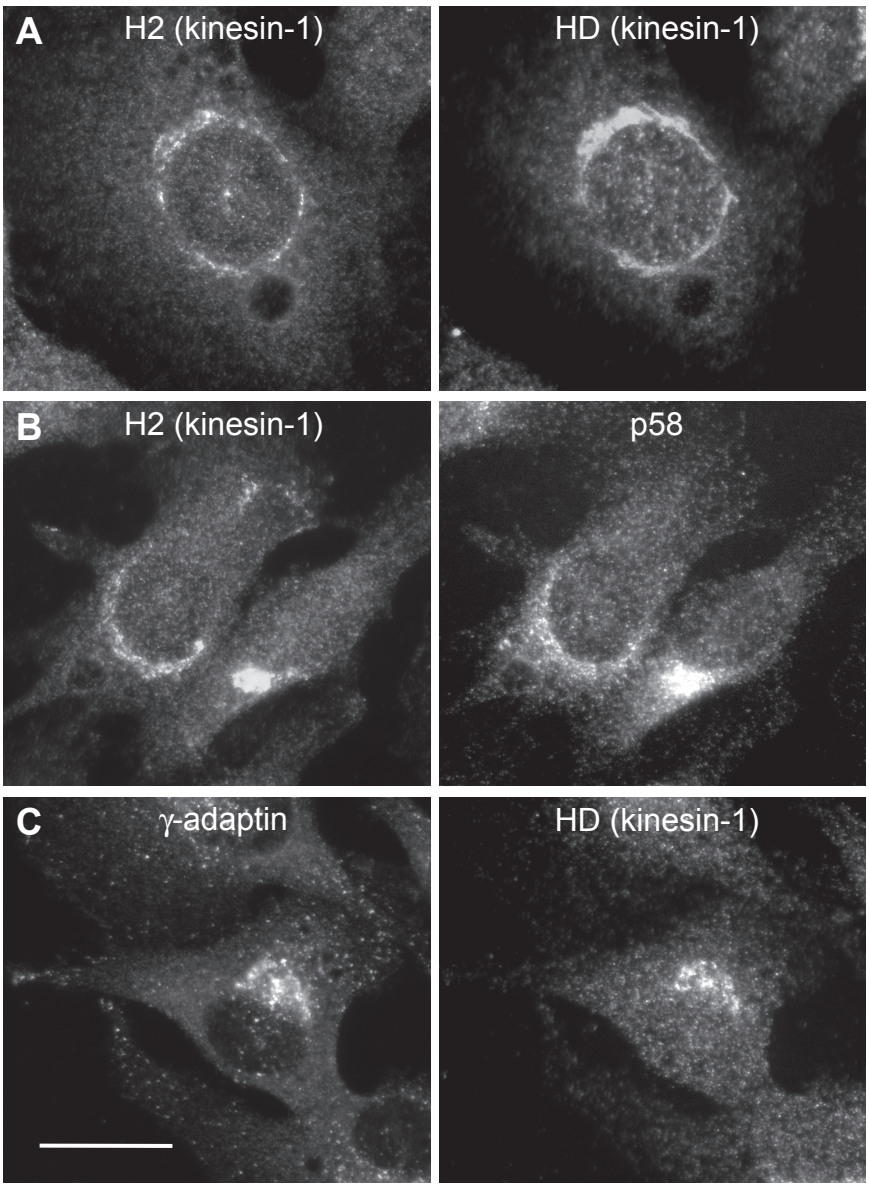
Supplementary figure 3



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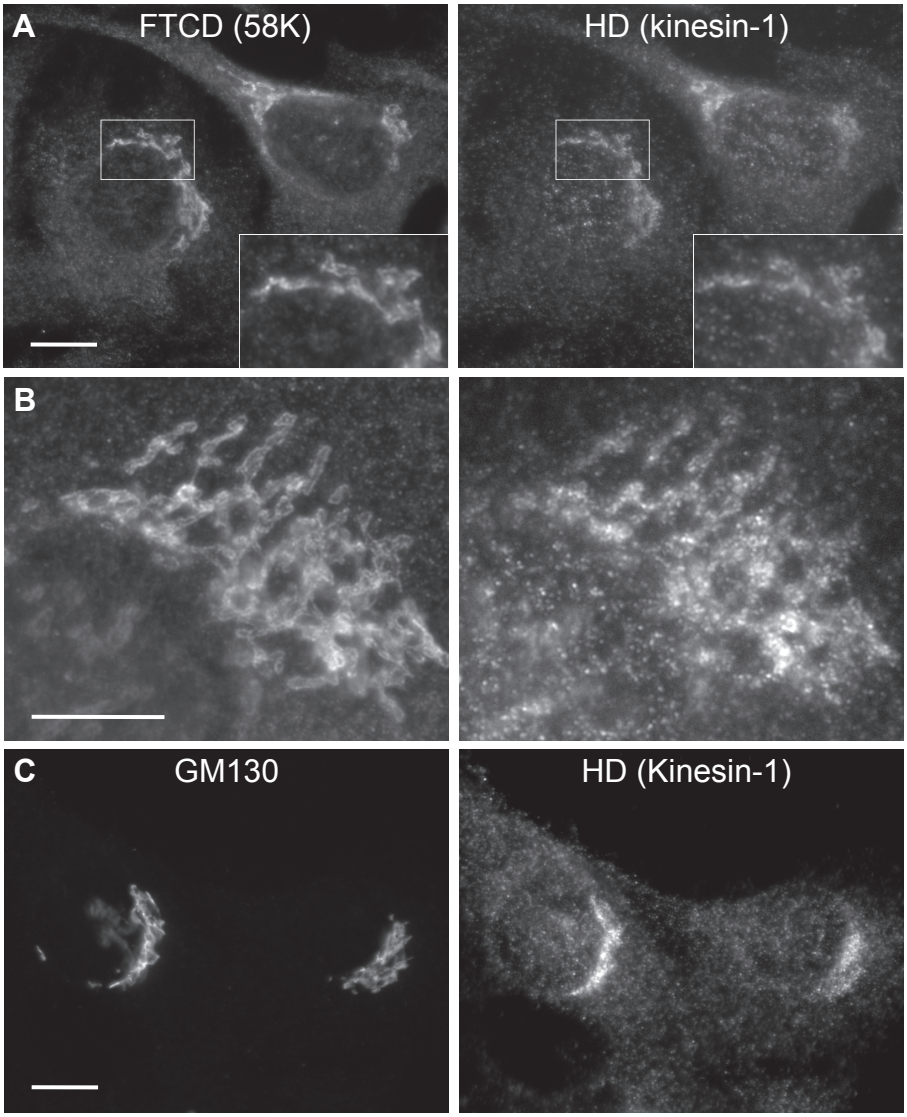
Supplementary figure 4



Supplementary Figure 4. Kinesin-1 colocalises with the Golgi-associated protein FTCD.
NRK cells fixed in methanol were labelled with the HD anti-kinesin antibody in combination with FTCD/58k (A, B) and GM130 (C) antibodies which label the Golgi apparatus. The insert in A is a 2.25 magnification of the selected region. Scale bars are 10 μm.