BMC Cell Biology

Research article

The XMAP215-family protein DdCP224 is required for cortical interactions of microtubules

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Abstract

Background: Interactions of peripheral microtubule tips with the cell cortex are of crucial importance for nuclear migration, spindle orientation, centrosome positioning and directional cell movement. Microtubule plus end binding proteins are thought to mediate interactions of microtubule tips with cortical actin and membrane proteins in a dynein-dependent manner. XMAP215-family proteins are main regulators of microtubule plus end dynamics but so far they have not been implicated in the interactions of microtubule tips with the cell cortex.

Results: Here we show that overexpression of an N-terminal fragment of DdCP224, the *Dictyostelium* XMAP215 homologue, caused a collapse of the radial microtubule cytoskeleton, whereby microtubules lost contact with the cell cortex and were dragged behind like a comet tail of an unusually motile centrosome. This phenotype was indistinguishable from mutants overexpressing fragments of the dynein heavy chain or intermediate chain. Moreover, it was accompanied by dispersal of the Golgi apparatus and reduced cortical localization of the dynein heavy chain indicating a disrupted dynein/dynactin interaction. The interference of DdCP224 with cortical dynein function is strongly supported by the observations that DdCP224 and its N-terminal fragment colocalize with dynein and coimmunoprecipitate with dynein and dynactin.

Conclusions: Our data show that XMAP215-like proteins are required for the interaction of microtubule plus ends with the cell cortex in interphase cells and strongly suggest that this function is mediated by dynein.

Background

Interactions of peripheral microtubule plus ends with the cell cortex are of crucial importance for nuclear migration, spindle orientation, centrosome positioning and directional cell movement. Cortical dynein and dynactin components play an important role in mediating such interactions, in cooperation with a microtubule plus end complex consisting of a growing number of microtubule-associated proteins [1,2]. Only little is known about a role of the XMAP215-family (named after their *Xenopus* repre-

sentative) of microtubule-associated proteins in this process. The ubiquitous occurrence of these proteins in all kinds of organisms including plants suggests general and indispensable functions [3]. In addition to their role as promoters of microtubule elongation, further functions in microtubule growth and nucleation [4,5] and centrosome duplication [5,6] have been described. In most species, XMAP215-family proteins are elongated, monomeric molecules with a size of approximately 230 kDa [7]. By contrast, the yeast homologues (Stu2p in *S. cerevisiae*, dis1
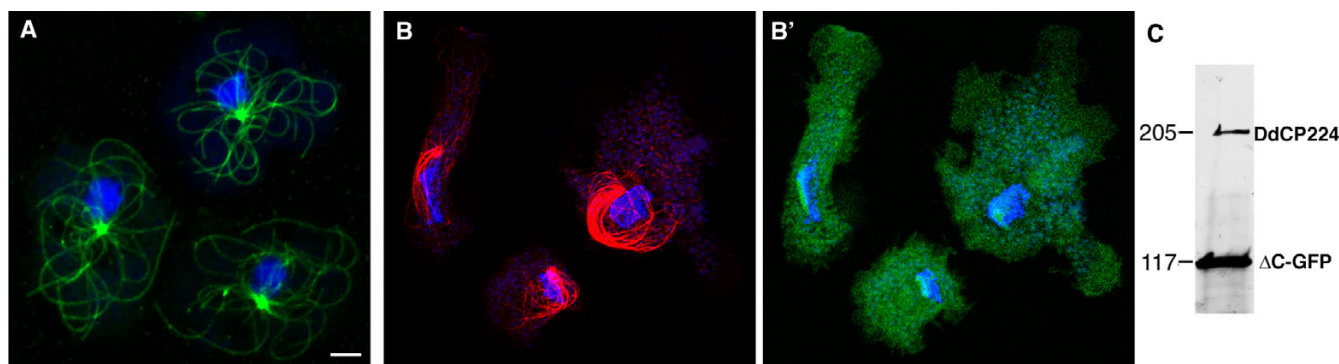
Figure 1
Overexpression of DdCP224-ΔC-GFP causes a collapse of interphase microtubule arrays. (A, B, B') Confocal microscopy of GFP-α-tubulin cells (A; control) and DdCP224-ΔC-GFP cells showing brightest point projections of GFP fluorescence (A, B') or immunofluorescence staining (B) using the YL1/2 anti-tubulin antibody (Chemicon, Hofheim, Germany). Cells were fixed with methanol. DNA was stained with TOPRO3 (Molecular Probes, Hilversum, Netherlands) (blue). (C) Immunoblot of a cytosolic extract of DdCP224-ΔC-GFP cells stained with anti-DdCP-HindIII. This rabbit polyclonal antibody was raised against the recombinant His-tagged N-terminus of DdCP224 using the 5'-HindIII fragment of the DdCP224 coding sequence. As calculated by the ImageJ program, DdCP224-ΔC-GFP is overexpressed approximately 5-fold. Bar 2 μm.

and Alp14 in *S. pombe*) occur as dimers and are less than half as long as their counterparts in *Dictyostelium* and higher cells [8-10]. In budding yeast the major function of Stu2p is observed during mitosis where it regulates microtubule dynamics and is required for chromosome segregation [11-13]. Furthermore, Stu2p interacts with the cortical protein Kar9p [13] and genetic evidence, i.e. crossings of temperature sensitive *stu2p* mutants with *kar9Δ* or dynein (*dhc1Δ*) mutants, suggests that Stu2p plays a role in the Kar9p dependent pathway for spindle orientation [12]. However, until this work there was no evidence for a physical interaction of the long, monomeric members of the XMAP215-family with dynein or a Kar9p-like protein such as APC [14], and there were no data supporting a role in microtubule plus-end/cell cortex interactions in interphase cells.

Like XMAP215, its *Dictyostelium* homologue, DdCP224, is both a microtubule-associated protein and a genuine centrosomal component [6,15]. Furthermore, it was the first member of the XMAP215-family that was clearly localized at microtubule plus ends, both at kinetochores and microtubule tips near the cell cortex [6,16]. Overexpression of the N-terminal half of DdCP224 as a GFP-fusion protein caused a cytokinesis defect [6]. Since cleavage furrow positioning is determined by the pattern of interaction of astral microtubules with the cell cortex [17], both the cytokinesis defect of ΔC-GFP overexpressing mutants and the detection of DdCP224 at microtubule tips were in agreement with a novel role of DdCP224 in the crosstalk of microtubule tips with the cell cortex. Here we provide

evidence for such a function of XMAP215-like proteins and suggest that it is mediated through the interaction with dynein.

Results

DdCP224-ΔC-GFP mutants overexpress a C-terminal fusion of the N-terminal 813 amino acids of DdCP224 with GFP. By contrast to the full-length protein, the DdCP224-ΔC-GFP fragment itself is unable to bind to microtubules or centrosomes neither *in vivo* nor *in vitro* [6]. In our previous study, we have not analyzed the effect of DdCP224-ΔC-GFP overexpression on microtubules. In this work we show that overexpression of DdCP224-ΔC-GFP has a profound effect on the arrangement of interphase microtubules. In wildtype or GFP-α-tubulin cells, all interphase microtubules emanate from the centrosome and are arranged in a radial array with their tips close to the cell cortex (Fig 1A). By contrast, in mutants overexpressing DdCP224-ΔC-GFP, these arrays were collapsed. Microtubules now frequently appeared bundled, were longer than usual and whorled around the nucleus (Fig 1B). Moreover, microtubule tips had lost contact with cortical regions. As calculated from Western blots, the DdCP224-ΔC-GFP fragment was overexpressed approximately 5-fold (Fig. 1C).

In order to investigate the behavior of these unusual microtubule arrays in living cells, we have transformed the untagged DdCP-ΔC fragment into *Dictyostelium* cells expressing GFP-α-tubulin. Four-dimensional confocal microscopy revealed an extraordinary motility of the
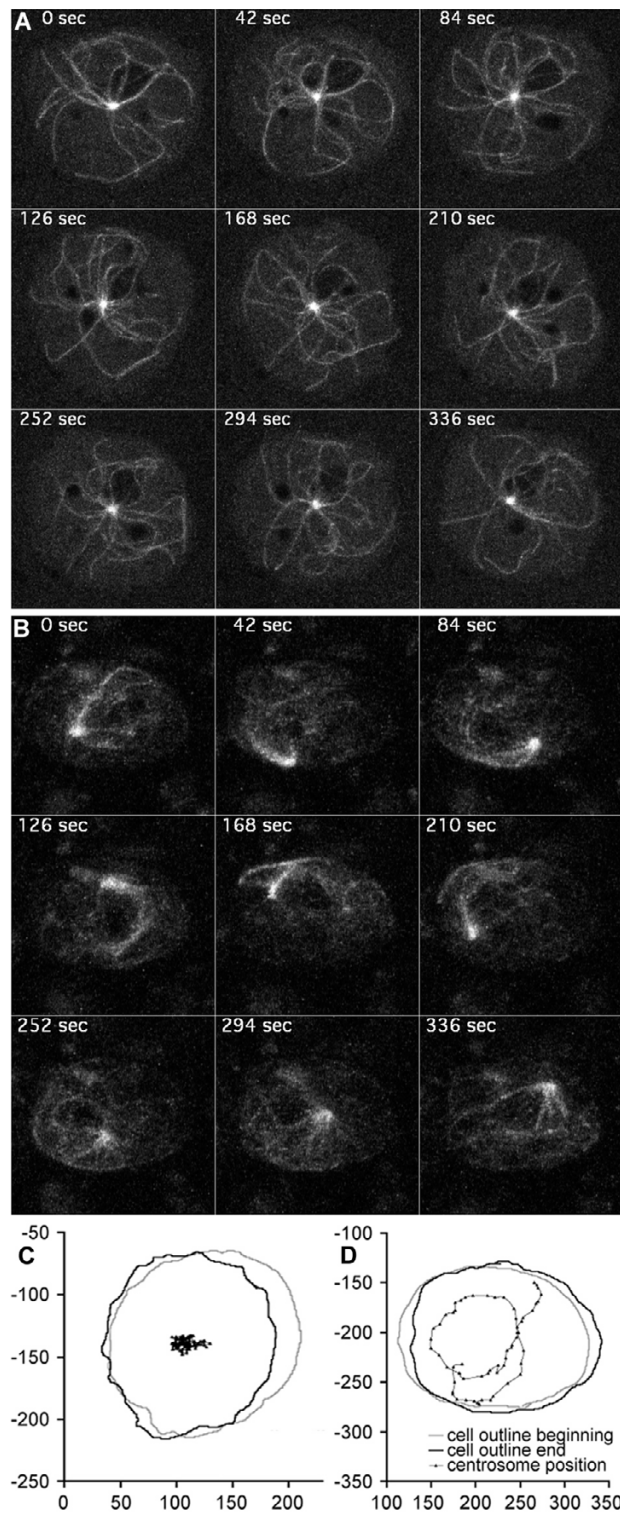
Figure 2
Microtubule and centrosome dynamics upon overexpression of DdCP224-ΔC. GFP-α-tubulin control cells (A, see additional data file movie1.mov) and GFP-α-tubulin cells overexpressing DdCP224-ΔC (B, see additional data file movie2.mov) were analyzed by confocal 4D-microscopy as described [5]. Each image represents a brightest point z-projection of 5 confocal slices with a distance of 1 μm each. The time is indicated in seconds. The movements of the centrosome shown in (C) for control cells and in (D) for DdCP224-ΔC/GFP-α-tubulin cells were calculated from 60 single images each using ImageJ.
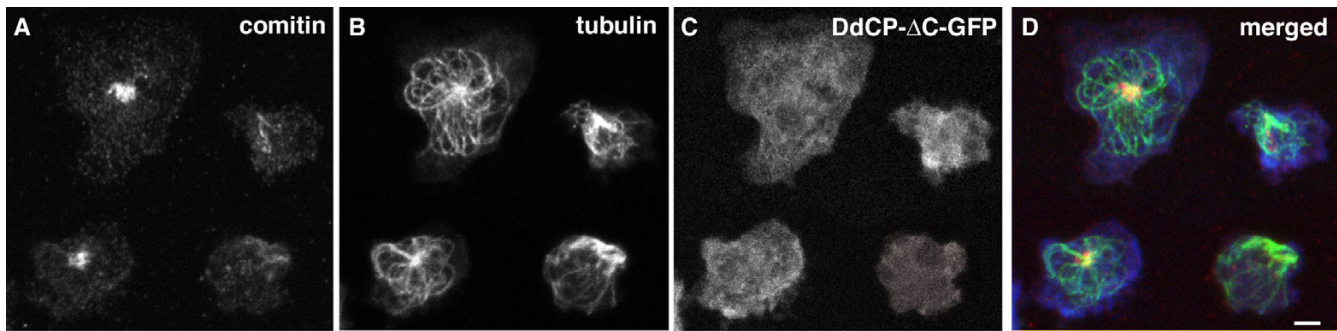
Figure 3
Cells with disrupted microtubule arrays show Golgi dispersal. Confocal microscopy of DdCP224-ΔC-GFP cells showing brightest point projections of immunofluorescence stainings using the Golgi-specific anti-comitin antibody [34] (A) and the YL1/2 anti-tubulin antibody (B). GFP fluorescence is shown in (C). The merged image (D) shows the Golgi in red, microtubules in green and GFP fluorescence in blue. Cells were fixed with formaldehyde/acetone. The two cells exhibiting Golgi dispersal and disrupted microtubule arrays are marked by an arrow. Bar 2 μm.

microtubule arrays. Microtubules often were arranged like a comet-tail attached to the centrosome. The centrosome itself circulated around rapidly and continuously, often close to the cell cortex (Fig. 2B,2D; see additional data file movie2.mov). In control cells, the centrosome always stayed close to the cell center and moved only short distances (Fig. 2A,2C; see additional data file movie1.mov). The phenotype upon overexpression of DdCP-ΔC was indistinguishable from *Dictyostelium* cells overexpressing the motor domain of the dynein heavy chain [18,19]. Interestingly, disruption of the dynein/dynactin interaction in *Dictyostelium* cells by overexpression of fragments of the dynein intermediate chain also resulted in this phenotype [20].

The striking similarity of the DdCP-ΔC mutants to these dynein mutants strongly suggested that our phenotype can also be attributed to a defect of dynein function, e.g. due to a disruption of dynein/dynactin interaction. Since this interaction is also crucial for positioning of the Golgi apparatus, we analysed this issue in our DdCP224-ΔC-GFP mutants. Indeed, cells with disrupted microtubule arrays often showed complete dispersal of the Golgi apparatus (Fig. 3), suggesting that overexpression of the N-terminal DdCP224 fragment causes dissociation of dynein and dynactin. This interpretation is also supported by the observation that such cells usually showed reduced cortical localization of the dynein heavy chain compared to cells which have maintained a radial microtubule array (Fig. 4).

The interference of DdCP224 with dynein function raised the question whether DdCP224 could interact with dynein. Although a major fraction of cytosolic DdCP224

occurs as a monomer [6], we could coimmunoprecipitate the dynein heavy and intermediate chains with DdCP224 from a cytosolic extract (Fig. 5A,5B). Vice versa, DdCP224 could be coimmunoprecipitated with both the dynein heavy and intermediate chains. Hence, a considerable fraction of DdCP224 occurs in cytosolic complexes with dynein and possibly other proteins. We cannot judge whether DdCP224 binds directly to dynein, since these proteins cannot be functionally expressed in *E. coli* for pull-down assays. The cytosolic complex consisting of DdCP224 and dynein may contain the *Dictyostelium* EB1 (DdEB1) [16] as well, since DdEB1 coprecipitates both with DdCP224 and dynein (Fig. 5C,5D).

DdCP224 does not only coprecipitate with dynein, it also colocalizes with the dynein heavy chain not only at the centrosome but also at the cell cortex at the leading edges and in pinocytic cups (Fig. 6A). The DdCP224-ΔC-GFP fragment also strongly colocalized with endogenous DdCP224 at these cortical regions (Fig. 6B). Visualization of the cortical localization of DdCP224 was overlooked in our earlier studies where the cells were fixed with methanol since it requires fixation with formaldehyde/acetone or glutaraldehyde.

To elucidate how overexpression of the DdCP224-ΔC-GFP fragment could cause the mutant phenotype, we investigated its intermolecular interactions. Since the DdCP224-ΔC-GFP fragment did not coimmunoprecipitate with either dynein or DdEB1 (data not shown), we wondered whether it might interact with dynactin. Due to the lack of specific antibodies against dynactin components, we cloned the *Dictyostelium* homologue of dynactin-p62 (Ddp62) as a marker for dynactin and
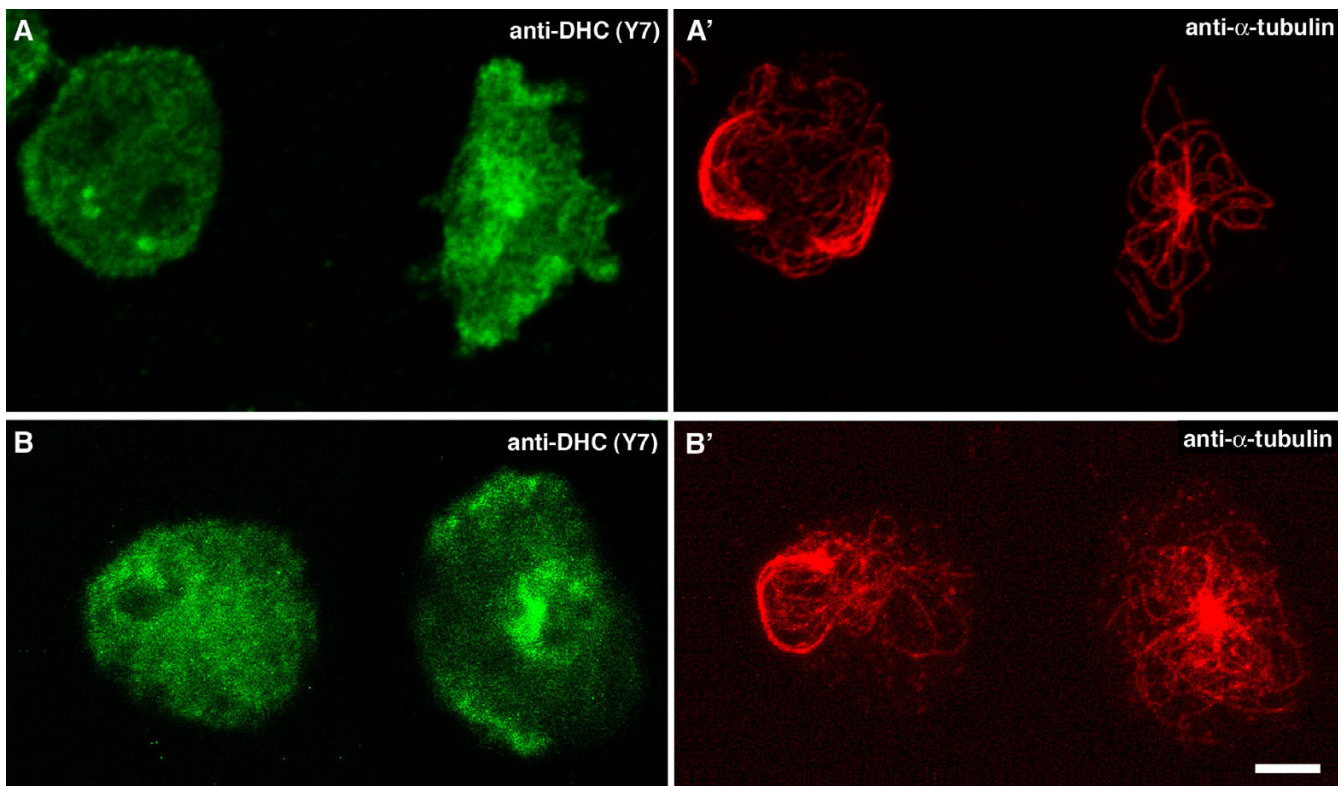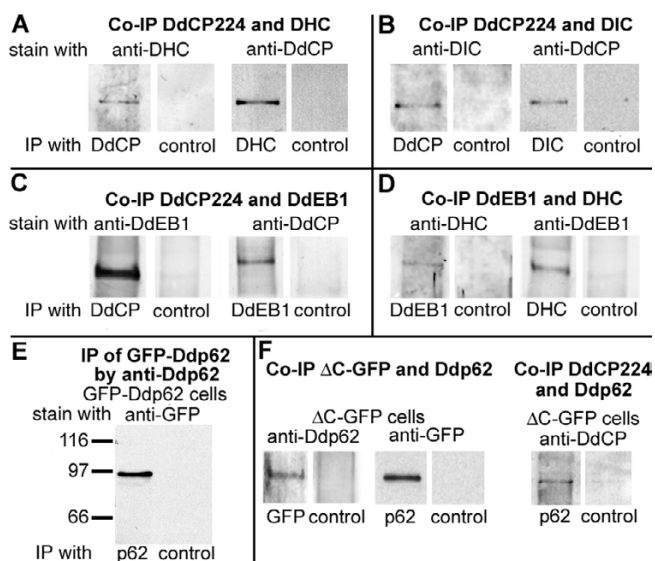
Figure 4
Cells with disrupted microtubule arrays show reduced cortical localization of dynein. Confocal microscopy of DdCP224-ΔC-GFP cells showing brightest point projections of immunofluorescence stainings using anti-dynein-Y7 [19] (A, B) and and the YL1/2 anti-tubulin antibody (A', B'). In both examples, the left cell exhibits a disrupted microtubule array and is characterized by reduced cortical distribution of the dynein heavy chain compared to the right cell which shows normal micro-tubules. Cells were fixed with formaldehyde/acetone. Bar 2 μm.

raised antibodies against the recombinant protein. These antibodies showed only weak staining of denatured Ddp62 in Western blots. However, they were capable of specific immunoprecipitation of a GFP-Ddp62 fusion protein from cytosolic extracts of *Dictyostelium* GFP-Ddp62 mutants (Fig. 5E). Hence, we concluded that these antibodies showed a higher avidity to native than to denatured Ddp62. Using these antibodies we could demonstrate by co-immunoprecipitation that Ddp62 binds to endogenous DdCP224 and to the cytosolic DdCP224-ΔC-GFP fragment (Fig. 5F).

Discussion

Centrosome movements and positioning are thought to be a result of balanced pulling forces that are transmitted through microtubules interacting with a cell cortex-associated motor protein. Dynein, which was recently localized to the cell cortex in *Dictyostelium* cells [21], provides such microtubule minus end-directed forces. These pulling forces are required for maintenance of the radial array of interphase microtubules and centrosome centering in wildtype cells (Fig. 7A). If most of the cortical dynein/dynactin complexes were dissociated or non-functional, cortical tethering of most microtubules would be lost. Yet, single microtubules remaining in contact with intact cortical dynein/dynactin complexes could rapidly be pulled to the cortex together with the centrosome and all the untethered microtubules that are dragged behind like a comet tail (Fig. 7B). An alternative explanation for this type of centrosome movement would be that the loss of minus-end directed forces could render microtubules more susceptible for pushing forces when they occasionally interact with cortically localized plus-end-directed motors [18]. However, the existence of such cortical plus-end directed motors remains to be shown, while the necessity of dynein and dynactin for centrosome positioning is undisputed, since it has also been proven in wound-healing experiments using fibroblast monolayers [22]. During the healing process, cells at the wound edge reorient their centrosomes toward the direction of migra-

Figure 5
Coprecipitation of DdCP224 with dynein and DdEB1.
Experiments were performed using cytosolic extracts from
wildtype cells (strain AX2) (A-D), GFP-Ddp62 cells (E) and
DdCP224-ΔC-GFP cells (F). The respective co-immunopre-
cipitation (Co-IP) experiment is given in bold letters on top
of each subfigure. The respective antibodies used for immu-
noprecipitation and staining of the immunoblots are indi-
cated. Abbreviations and antibodies: DHC, anti-dynein heavy
chain Y7 [19]; DIC, anti-dynein intermediate chain [20];
DdCP, anti-DdCP224 mAb 2/165 [35] for immunoblot stain-
ing and anti-DdCP-HindIII for immunoprecipitation; DdEB1,
anti-DdEB1 [16]; p62, anti-Ddp62; control, anti-rabbit or
anti-rat (in case of DIC) preimmune serum.

tion [23]. Centrosome reorientation between the leading
edge of the cell and the nucleus is blocked by inhibition
of cytoplasmic dynein and dynactin and regulated
through the small GTPase Cdc42 and PKCζ [24,25]. This
suggests that cortical dynein/dynactin is required for cap-
turing of microtubules extending into cortical regions of a
freshly formed pseudopod.

What could be the role of the cortical and the microtubule
tip populations of DdCP224 [16] in microtubule/cortex
interactions? At microtubule tips it could play a role in the
capturing of microtubule plus ends at cortical sites, how-
ever, since DdCP224, like XMAP215, promotes microtu-
bule growth [5], it is likely that the major function of tip-
localized DdCP224 is the regulation of microtubule plus-
end dynamics and the prevention of catastrophes induced
by antagonistic Kin I-family kinesins [26]. By contrast, the
cortical population of DdCP224 that colocalizes with
dynein appears to be required for proper dynein function
at the cortex. This is supported by two observations. First,

DdCP224 binds to dynein/dynactin and second, overex-
pression of the DdCP224-ΔC-fragment disrupts cortical
dynein function. Excess amounts of this DdCP224 frag-
ment appear to interfere with the interaction between
dynein and dynactin, since the characteristic collapse of
the radial microtubule array was accompanied by reduced
cortical dynein localization and by Golgi dispersal which
is indicative for disrupted dynein/dynactin interaction
[27]. The simplest explanation is that the DdCP224-ΔC-
fragment sequesters Ddp62 and possibly other dynactin
components in the cytosol which are then missing at the
cell cortex where they are required for proper dynein func-
tion. The cytokinesis defect observed upon overexpression
of the DdCP224-ΔC-GFP fragment [6] also agrees with an
active role of DdCP224 in the interaction of microtubule
tips with cortical sites, since these interactions are
involved in cleavage furrow positioning [17].

The pathway of dynein/dynactin/DdCP224-dependent
cortical interactions of interphase microtubules reported
herein has to be distinguished from that of spindle
orientation in mitotic yeast cells. In the latter case, Stu2p
is involved in Kar9p-dependent capture of cytoplasmic
MT plus-ends at the bud tip [12,13], a process that essen-
tially requires yeast EB1 (Bim1p) at the MT tips [28,29].
Although *Dictyostelium* EB1 interacts with both DdCP224
and dynein, the process of MT/cortex interaction
described here is clearly independent of DdEB1. DdEB1
null mutants showed only a defect in mitotic spindle for-
mation, but neither a defect in microtubule organization
or centrosome positioning, nor a cytokinesis defect [16].

Conclusions
Taken together, our results demonstrate for the first time
that XMAP215-family proteins such as DdCP224 are
involved in microtubule plus-end/cell cortex interactions
and centrosome positioning in interphase cells and that
this is mediated through an interaction of DdCP224 with
dynein and dynactin.

Methods
### Generation of the GFP-α-tubulin/DdCP224-ΔC mutant
The *Dictyostelium* vector for expression of the untagged N-
terminal 813 amino acids of DdCP224 was constructed by
deletion of the GFP sequence in pΔC-GFP [6]. It was then
transformed into a *Dictyostelium* cell line expressing GFP-
α-tubulin [16]. Cells were cultured as described earlier [6].

Cloning of Ddp62, protein expression and generation of polyclonal antibodies
The gene encoding the *Dictyostelium* homologue of the
p62 subunit of dynactin (Ddp62; DictybaseID
DDB0206421) was identified in the *Dictyostelium* genome
project [30]. Its complete coding sequence (1647 bp) was
amplified by PCR using an oligo dT-primed cDNA library
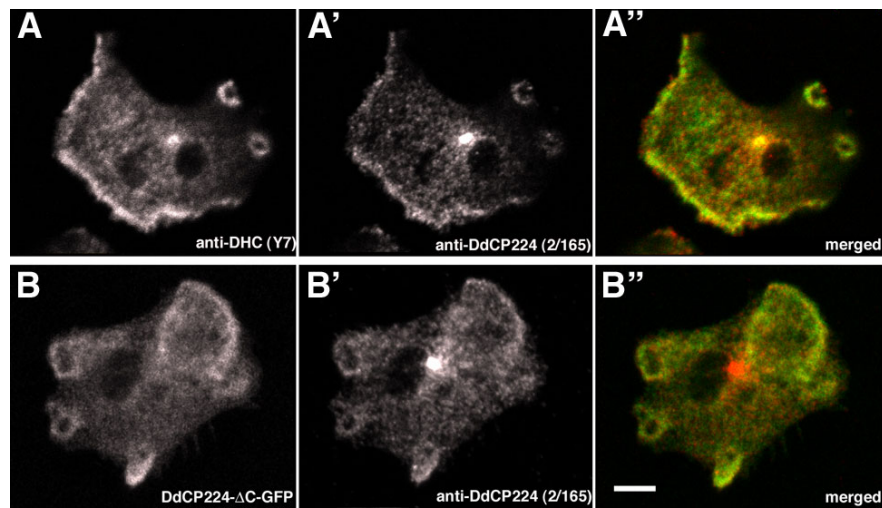
Figure 6
Colocalization of DdCP224, DdCP224-ΔC-GFP and dynein at the cell cortex. Confocal microscopy of DdCP224-ΔC-GFP cells showing brightest point projections of immunofluorescence stainings using anti-dynein-Y7 [19] (A) and anti-DdCP224 (2/165) (A', B'). GFP fluorescence is shown in (B). The merged images (A", B") shows endogenous DdCP224 in red and dynein (A) or DdCP-ΔC-GFP (B'), respectively, in green. Cells were fixed with formaldehyde/acetone. Bar 2 μm.
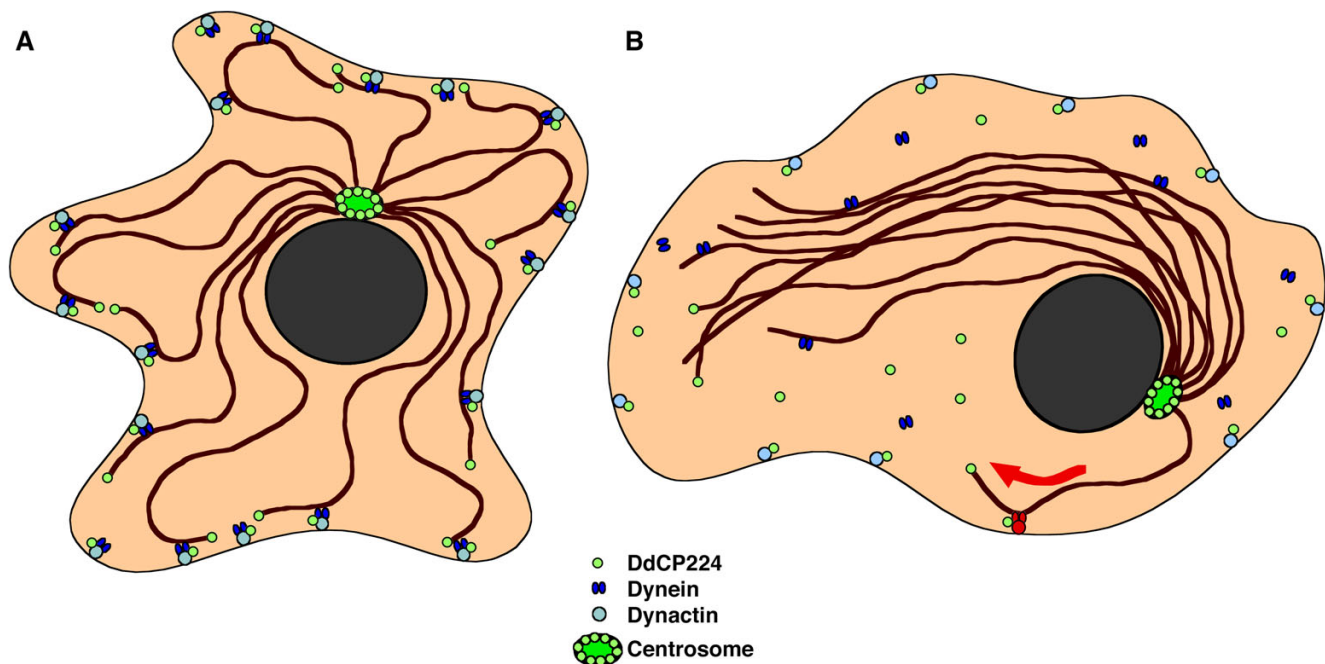
Figure 7
Model for the collapse of radial interphase microtubule arrays by disruption of cortical dynein/dynactin function. (A) Cortical dynein/dynactin in cooperation with DdCP224 provides the pulling force for maintenance of radial microtubule arrays. (B) Collapse of the radial microtubule array and altered centrosome positioning due to disruption of most cortical dynein/dynactin complexes and asymmetric pulling forces provided by only a few remaining functional cortical dynein/dynactin complexes (shown in red). This pathway may also involve further proteins which are not depicted in this model.

[6] as a template. The Ddp62 cDNA was re-amplified using either BamHI and PstI linker primers for cloning into the pMALc2 (NEB, Frankfurt, Germany) or SalI and BamHI linker primers for cloning into pIS77, a vector obtained after replacement of the discoidin promoter of pDiscGFPSSEB2 [31] by the actin6 promoter. The former construct was used for protein expression in *E. coli*, the latter for expression of a GFP-Ddp62 fusion protein in *Dictyostelium*. The MBP-Ddp62 fusion protein expressed in *E. coli* was purified by affinity chromatography on amylose resin and used for custom immunization of two rabbits (Pineda Antikörperservice, Berlin, Germany). Both antisera showed the same characteristics.

Immunoprecipitation experiments

Immunoprecipitation was performed essentially as described previously [32]. In brief, 2×10^8 cells (80 ml) were lysed in 5 ml of lysis buffer (50 mM Hepes, 100 mM NaCl, 4 mM EGTA, 2 mM $MgCl_2$, 10% sucrose, 0.3% NP40, 1 × protease inhibitor cocktail [33]). A cytosolic extract was obtained after centrifugation at 14.000 × g for 10 min at 4°C. After incubation of 0.6 ml of cytosolic extract with 10 μg of purified antibodies or 1.5 μl of antiserum for 1 h at 4°C, 20 μl of Protein G beads (50% slurry preincubated with 0.1% BSA in Tris-buffered saline) were added for a further incubation for 1 h at 4°C in a rotator. Beads were washed 4 times with lysis buffer, resuspended with 30 μl of SDS sample buffer (10% SDS, 125 mM Tris/HCl, pH = 6.8, 50 mM DTT, 5% glycerol) and subjected to electrophoresis and Western blotting as described previously [33].

Microscopy and image processing

Immunofluorescence microscopy and live cell observation was performed as described previously [5,33]. All microscopic images were acquired on a Zeiss Axiovert 200M/510META confocal microscope equipped with a 63x/1.4N.A. lens.

Authors' contributions

AH performed confocal microscopy of living cells and of most fixed immunofluorescence specimens. RG has cloned Ddp62, made protein purifications and carried out the immunoprecipitations. The manuscript was written by RG. AH has read and approved the final manuscript.

Additional material

Additional File 1

Microtubule and centrosome dynamics in GFP-α-tubulin control cells. Cells were viewed by confocal 4D-microscopy as described [5]. Each single image represents a brightest point z-projection of 5 confocal slices with a distance of 1 μm each. The time is indicated in seconds.
Click here for file
[http://www.biomedcentral.com/content/supplementary/1471-2121-5-24-S1.mov]

Additional File 2

Microtubule and centrosome dynamics upon overexpression of DdCP224-ΔC. GFP-α-tubulin control overexpressing DdCP224-ΔC were viewed by confocal 4D-microscopy as described [5]. Each single image represents a brightest point z-projection of 5 confocal slices with a distance of 1 μm each. The time is indicated in seconds.
Click here for file
[http://www.biomedcentral.com/content/supplementary/1471-2121-5-24-S2.mov]

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