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**A DOMINANT NEGATIVE ESCRT-III PROTEIN PERTURBS
CYTOKINESIS AND TRAFFICKING TO LYSOSOMES.**

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Running title: ESCRT-III and cytokinesis

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KEYWORDS: Abscission; Cytokinesis; Endosome; ESCRT; Midbody

¹ABBREVIATIONS: Charged multivesicular body protein, CHMP; Early endosomal
autoantigen 1, EEA1; Endosomal sorting complex required for transport, ESCRT;
Multivesicular body, MVB.

SUMMARY

In eukaryotic cells, the completion of cytokinesis is dependent on membrane trafficking events to deliver membrane to the site of abscission. Golgi and recycling endosomal derived proteins are required for the terminal stages of cytokinesis. Recently, protein subunits of the Endosomal Sorting Complex Required for Transport (ESCRT) that are normally involved in late endosome to lysosome trafficking have also been implicated in abscission. Here we report that a subunit, Charged Multivesicular Body Protein-3 (CHMP3), of ESCRT-III localises at the midbody. Deletion of the C-terminal autoinhibitory domain of CHMP3 inhibits cytokinesis. At the midbody, CHMP3 does not co-localise with Rab11 suggesting that it is not present on recycling endosomes. These data combined provide compelling evidence that proteins involved in late endosomal function are necessary for the end stages of cytokinesis.

INTRODUCTION

Proteins that make up the endosomal sorting complex required for transport (ESCRT)¹ are involved in the sorting and trafficking of membrane proteins into multivesicular bodies (MVBs). Current models of ESCRT function place the ESCRT proteins into three complexes, ESCRT-I, II and III (reviewed in [1-4]). ESCRT-I is thought to be involved in the recognition of ubiquitylated cargo membrane proteins that are to be sorted into MVBs. ESCRT-II and ESCRT-III have been implicated in further protein sorting and invagination of the endosomal membrane away from the cytoplasm to form MVBs. Once assembled on membranes, removal of ESCRT proteins by an AAA-ATPase called Vps4 is required in order for ESCRT proteins to carry out multiple rounds of sorting [5]. The latter stages of MVB formation are not currently well understood, however it is known that the ultimate fate of cargo proteins that are sorted into MVBs is usually degradation in lysosomes. Perturbing the function of the ESCRT machinery in mammalian cells by protein knockdown or expression of dominant negative proteins such as an ATPase defective Vps4 (Vps4^{E235Q}) results in intracellular accumulation of cargo proteins that fail to get degraded in lysosomes [6-8].

In addition to their role in sorting of membrane proteins for destruction in lysosomes, ESCRT proteins have been implicated in membrane virus budding (reviewed in [9]), mRNA trafficking [10] and cytokinesis [11-13]. The involvement of ESCRT proteins in cytokinesis is particularly interesting as evidence is accumulating from model organisms that endocytosis and many proteins involved in endocytic pathways such as dynamin, clathrin and Rab11 are also essential for the successful completion of cytokinesis [14-18].

Cytokinesis is the separation of one cell into two daughter cells following mitosis. During the final stages of cytokinesis in animal cells a midbody is formed which is a thin membrane tether-like structure connecting the two daughter cells together. The end of cytokinesis is typified by the abscission of this structure at the midbody, which is an event heavily reliant on membrane dynamics. A model for abscission that seems to unify the seemingly different mechanisms of cytokinesis in animals and plants, involves the trafficking of membrane vesicles to the midbody and phragmoplast, respectively [14, 19, 20]. At the mid-body, in animal cells, these vesicles have been suggested to fuse homotypically and heterotypically with the plasma membrane causing abscission at this region and separation of the two daughter cells [21]. The source of the membrane vesicles for abscission is seemingly complex, as proteins usually associated with Golgi, early stages of endocytosis and endosomal recycling all appear to be important in the process [14]. The recent discovery that ESCRT proteins, which are generally considered as functioning in late endosomal membrane trafficking, are required for cytokinesis adds additional complexity to the source of membranes present at the site of abscission. An ESCRT-I protein (TSG101), an ESCRT related protein (Alix) and ESCRT-III proteins (CHMPs 2, 4 and 5) are all present at the midbody during the final stages of cytokinesis in mammalian cells [11, 12]. Furthermore, interfering with the expression, by knockdown or overexpression, of TSG101, Alix and other ESCRT proteins results in impaired cytokinesis [11, 12]. Thus, it seems that a functional ESCRT machinery is required for the late stages of cytokinesis.

Following on from this work, we wanted to investigate whether the ESCRT-III protein CHMP3 is present at the midbody and functionally required for cytokinesis in animal cells. To address this point we utilised a dominant negative derivative of charged multivesicular body protein 3 (CHMP3), an ESCRT-III protein. We and others have shown that full length CHMP3-FLAG and -GFP fusion proteins are cytosolic, do not noticeably affect endosome morphology and do not inhibit membrane trafficking to lysosomes or HIV particle production when expressed in cultured mammalian cells [22-24]. However, truncated CHMP3-fusion proteins, with the C-terminal autoinhibitory domain removed, become membrane associated and act as dominant negative proteins in that they dramatically alter endosome morphology, prevent trafficking to the lysosome and inhibit virus budding [22-25]. We now show that as well as perturbing endocytic trafficking to the lysosome a dominant negative CHMP3 derivative (CHMP3¹⁻¹⁷⁹GFP) localises to the mid-body of dividing mammalian cells and dramatically inhibits cytokinesis.

EXPERIMENTAL PROCEDURES

DNA Manipulations and Constructs

A cDNA fragment encoding amino acid residues 1-179 of rat CHMP3 was cloned into the BglIII/HindIII sites of pEGFP-N1 (Clontech) as a BglIII/HindIII fragment to create a vector for the expression of the fusion protein CHMP3¹⁻¹⁷⁹GFP. Site directed mutagenesis was performed using the Quikchange method (Stratagene). The mutant protein CHMP3¹⁻¹⁷⁹GFP^{M1} had amino acids R24, K25, and R28 of CHMP3 changed to S, A and N respectively. All other constructs have been described previously [24, 26].

Antibodies

Mouse monoclonal anti- β -tubulin antibodies (2.1) were purchased from Sigma, anti-ubiquitin (FK2) from Biomol and anti-Rab1 1a from BD Biosciences. The rabbit anti-EEA1 (early endosome antigen 1) was a gift from Dr Michael Clague (University of Liverpool, Liverpool, U.K.), and the rabbit anti-CI-M6PR was a gift from Dr Paul Luzio (University of Cambridge, Cambridge, U.K.). Species-specific fluorophore (Alexa FluorTM 546)-conjugated anti-IgG secondary antibodies were all purchased from Molecular Probes.

Cell culture and Transfections

Cos-7 and HeLa cells were maintained at 37°C, 5% CO₂ in DMEM media supplemented with 10% (v/v) fetal calf serum, 2mM L-glutamine, 100units/ml penicillin and 100 μ g/ml streptomycin. Cells were plated onto 13mm coverslips in 24-well plates (Nunc) and grown until approximately 60% confluent when they were transfected with Trans-IT (Mirus) according to manufacturer's instructions.

Immunofluorescence and Multinucleation Counts

24 h post-transfection cells were fixed with 4% (w/v) paraformaldehyde for 20 min and permeabilised using methanol at -20°C for 5 min and then blocked with 10% newborn calf serum. Primary and secondary antibodies were diluted in 2% NCS-PBS (2% newborn calf serum in PBS) and cells were incubated with primary antibodies for ~ 2 h at 18°C and ~1 h for secondary antibodies. Cells were washed five times for 5 min with 2% NCS-PBS following all antibody incubations. Stained cells were then mounted in Mowiol (Calbiochem, San Diego, CA, U.S.A.) and examined on a Zeiss LSM510 laser scanning confocal microscope and appropriate images taken. For cell multinucleation counts, transfected cells were counted on a coverslip and scored for either a single nucleus or multiple (two or more) nuclei. Cells with continuous plasma membrane and connected by tethered tubulin "bridges" between them were defined as multinucleated, provided both "cells" contained nuclei.

EGF Degradation Assay

HeLa cells were seeded on 13 mm coverslips 24 h prior to transfection and grown to 60-80% confluency. Cells were transfected using TransIT reagent as described, with CHMP3¹⁻¹⁷⁹GFP or CHMP3¹⁻¹⁷⁹GFP^{M1} and allowed to express the constructs for 24 h. The media was replaced, following a wash in warm PBS, with DMEM containing 1% BSA (no FBS). Cells were serum starved in this media for 16 h and then incubated with 500ng/ml Alexa FluorTM 555-conjugated EGF (Invitrogen) for 2 min or 60 min. Following EGF stimulation for given times, cells were washed twice with cold PBS and then fixed in PFA, stained with DAPI for 30 min and mounted onto slides using Mowiol. Slides were examined on a Zeiss LSM510Meta laser scanning confocal microscope.

RESULTS

The Dominant Negative Protein CHMP3¹⁻¹⁷⁹ is Present at the Midbody During Cytokinesis.

Recent work has shown that TSG101, an ESCRT-I component, localises to Flemming bodies during the late stages of cytokinesis and that its knockdown by siRNA inhibits cytokinesis at abscission [12]. In the same study it was shown that ESCRT-III components may also have a role to play in abscission. In another study the ESCRT-III proteins CHMP2, 4 and 5 have been localised to the midbody of dividing cells [11]. In order to investigate this further and determine whether other ESCRT-III components are also present at the midbody during the final stages of cytokinesis we made use of a dominant negative CHMP3 construct (CHMP3¹⁻¹⁷⁹GFP). Transient transfection of this dominant negative truncated form of CHMP3 resulted in a swollen vacuolar phenotype typified by CHMP3¹⁻¹⁷⁹GFP bound to large vacuolar structures in Cos-7 (Fig. 1) and HeLa cells (not shown). These structures were endosomal in origin as they contained both early and late endosomal markers (EEA1 and M6PR) (Fig. 1a-f). Ubiquitylated proteins also accumulated on the CHMP3¹⁻¹⁷⁹GFP containing endosomes (Fig. 1g-i). It is possible that the accumulated ubiquitylated proteins are cargoes destined for lysosomal degradation, however we cannot rule out the possibility that they are ubiquitylated cytoplasmic proteins recruited to membranes or even ESCRT components. In an EGF degradation assay fluorescent EGF accumulated intracellularly after 60 minutes incubation with EGF in CHMP3¹⁻¹⁷⁹GFP expressing cells but disappeared almost completely from neighbouring untransfected cells (Fig. 2d-f). These results indicate that the CHMP3¹⁻¹⁷⁹GFP fusion protein is dominant negative, as its expression blocks trafficking of EGF to the lysosome and prevents its degradation.

In cells fixed during the late stages of cytokinesis, the midbody was observed to contain CHMP3¹⁻¹⁷⁹GFP protein apparently present on membrane vesicles (Fig. 3a-c). The CHMP3¹⁻¹⁷⁹GFP specifically localises to the central region of the midbody, where there is

lack of β -tubulin staining. This distribution was observed in both Cos-7 cells (Fig. 3a-c) and HeLa cell lines (Fig. 3d-f), indicating that this phenomenon is not cell type specific. CHMP3¹⁻¹⁷⁹GFP was often seen distributed along the microtubules in the midbody channel (not shown) suggesting that vesicles may be being transported along microtubules towards the midbody. Interestingly, although Rab11, an endosomal protein required for abscission [27], was present in the midbodies of dividing cells, it did not seem to co-localise with CHMP3¹⁻¹⁷⁹GFP (Fig. 3g-i).

Dominant Negative CHMP3 Perturbs Cytokinesis.

HeLa cells were transfected with constructs for the expression of CHMP3¹⁻¹⁷⁹GFP, GFP-Vps4^{E235Q} and GFP-Vps4^{WT} or a GFP control. They were then fixed and immunostained for β -tubulin and treated with DAPI to stain nuclei. Transfected cells were quantified under the fluorescence microscope for the percentage of multinucleate cells. Multinucleate cells were defined as cells connected by a continuous plasma membrane to another cell, thus containing two or more nuclei (Fig. 4b). Of the GFP control transfected cells, 12% were multinucleate (Fig. 4a). The percentage of multinucleate cells was dramatically increased (to 48%) in CHMP3¹⁻¹⁷⁹GFP expressing cells. This indicates that CHMP3¹⁻¹⁷⁹GFP acts as a dominant negative protein in cytokinesis in addition to lysosomal trafficking. In agreement with a previous study [12], the positive control, GFP-Vps4^{E235Q} blocked cytokinesis, while GFP-Vps4^{WT} had very little effect. GFP-Vps4^{E235Q} appears to block cytokinesis at a late stage, similar to CHMP3¹⁻¹⁷⁹GFP, as it is also enriched on vesicles at the midbody of dividing cells (data not shown). The ATPase activity of Vps4 which is required to disassemble ESCRT-III from membranes seems therefore to be required for abscission. GFP-Vps4^{WT}, which does not block cytokinesis, is not enriched at the midbody.

In a recent study, Muziol *et al* [28] showed that the dominant negative effect of a truncated CHMP3 construct on viral budding could be abrogated by mutating 3 basic amino acids (R24, K25, and R28). We mutated these same amino acids to create CHMP3¹⁻¹⁷⁹GFP^{M1} and expressed this protein in mammalian cells. Unexpectedly CHMP3¹⁻¹⁷⁹GFP^{M1} associated with endosomal membranes (Fig. 5a,b), caused the intracellular accumulation of ubiquitylated proteins (Fig. 5c), internalised EGF (Fig. 2g-h) and blocked cytokinesis (Fig. 4 and 5d). Thus, mutations that abrogate the dominant negative effect of truncated CHMP3 on viral budding do not block the dominant negative effect of truncated CHMP3 on trafficking to the lysosome or cytokinesis.

DISCUSSION

Vesicular membrane traffic is important for the successful completion of cytokinesis in animal cells and in plants [14, 19, 20]. In plants, membrane vesicles provide material for cell plate formation and in animals membrane trafficking is required for midbody channel closure. The source of membranes that are targeted to the midbody channel is seemingly complex, with Golgi associated proteins involved in exocytosis and proteins that control endocytic recycling being required for late stages of cytokinesis. Recently, three studies, one in plants, and the others in mammalian cells have implicated that proteins best characterised as being involved in MVB biogenesis are also required for cytokinesis [11-13].

In this study we show that a dominant negative ESCRT-III protein, CHMP3¹⁻¹⁷⁹GFP localises to the midbody in dividing cells. As TSG101, an ESCRT-I component localises to the Flemming body [12], a phase dense structure involved in abscission, and the ESCRT-III proteins CHMP2, 4 and 5 localise to the midbody of dividing cells [11], our data strengthens the hypothesis that a complete ESCRT machinery may be present at the midbody. We further show that expression of CHMP3¹⁻¹⁷⁹GFP inhibits cytokinesis, as seen by a large increase in the percentage of multinucleate cells compared to controls. This indicates that the ESCRT-III machinery is not simply passively present at the site of abscission but is also functionally required.

So what is the role of the ESCRT machinery in abscission? It has been suggested that the ESCRT machinery may be involved in cytokinesis at the step of membrane fission as it is likely that ESCRT protein function in cytokinesis, virus budding and MVB formation is mechanistically conserved [12]. To support this, ESCRT proteins, in particular those in ESCRT-III are required for a late stage, possibly fission, in virus budding [29-31]. This process is topologically similar to the separation (budding) of two cells and the scission of inwardly budded vesicles from the limiting membrane of late endosomes to form MVBs. However, it has recently been shown that CHMP3 is not absolutely required for intraluminal vesicle formation at late endosomes [6]. Therefore it has been proposed that CHMP3 is important for the fusion of multivesicular endosomes with lysosomes [6] and not fission of inwardly budded endosomal vesicles. This raises the intriguing possibility that CHMP3 may be involved not in membrane fission but in vesicle fusion events that take place at the site of abscission [14, 21, 32]. A further possibility is that the ESCRT machinery is involved in the function of recycling endosomes that are required for cytokinesis [27, 33, 34]. Several studies have shown that perturbation of ESCRT function results in a defect in endosomal recycling [7, 8]. However, although we observe some co-localisation of CHMP3¹⁻¹⁷⁹GFP with Rab11 on endosomes in the cytoplasm, there is little overlap of CHMP3¹⁻¹⁷⁹GFP with Rab11 at the midbody (Fig. 3g-i). This suggests that there are at least two distinct populations of endosomal membranes, late and recycling endosome derived membranes, in addition to Golgi

derived membranes, present at the site of abscission. The complexity of components present at the midbody that are necessary for abscission highlights the sophistication of the mechanisms required for the final stage of cytokinesis. Much work is still required to understand these final stages.

In order to investigate whether membrane association of CHMP3 is required for localisation to the midbody and inhibition of cytokinesis, we assessed the effect of the mutant CHMP3¹⁻¹⁷⁹GFP^{M1} on cytokinesis. CHMP3 with the same 3 positively charged amino acid residues mutated had previously been shown to lose both its ability to associate with membranes and its dominant negative effect on viral budding [28]. CHMP3¹⁻¹⁷⁹GFP^{M1} localised at the midbody (Fig. 5d) and inhibited cytokinesis, although to a slightly lesser extent than the non-mutated protein (Fig. 4). On further analysis it was observed that unexpectedly CHMP3¹⁻¹⁷⁹GFP^{M1} clearly associated with endosomal membranes and also perturbed trafficking to the lysosome as indicated by the accumulation of ubiquitylated cargo proteins and internalised EGF in cells expressing CHMP3¹⁻¹⁷⁹GFP^{M1} (Fig. 2g-i and 5). Thus, it was not possible to determine whether membrane association is required for localisation to the midbody and inhibition of cytokinesis. In their study, Muziol *et al* [28] observed plasma membrane association of truncated CHMP3-GFP constructs, whereas we (Fig. 1 and data not shown) and others [23] do not, except at very high expression levels (data not shown). We have previously shown that CHMP3 binds to the endosomal lipid phosphatidylinositol 3,5-bisphosphate *in vitro* and have argued, as have others, that over-expressed, truncated CHMP3 may associate with membranes via endosomal specific lipids rather than protein-protein interactions [23, 24]. It will be interesting to determine whether endosome-specific lipids are required for abscission as establishment of specialised lipid composition seems to be important in cytokinesis [14]. It is difficult to explain the discrepancies in membrane association of the CHMP3 mutant constructs between our study and Muziol *et al* [28], but it is possible that cytokinesis and trafficking to the lysosome are more sensitive to ESCRT-III perturbation than viral budding.

In summary, we have shown that a dominant negative ESCRT-III protein, CHMP3¹⁻¹⁷⁹GFP localises to the midbody and inhibits cytokinesis at a late stage. It is likely that the ESCRT machinery is involved in abscission but further studies will be required to resolve the detailed mechanism of the role of CHMP3 in cytokinesis.

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Stage 2(a) POST-PRINT

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FIGURE LEGENDS

Fig. 1 Transient expression of CHMP3¹⁻¹⁷⁹GFP results in localisation to early and late endosomal compartments as well as impaired trafficking. Cos-7 cells were transfected (*a-i*) with the CHMP3¹⁻¹⁷⁹GFP fusion construct using Trans-IT transfection reagent according to manufacturer's instructions. Cells were fixed 24 h after transfection and immunostained with anti-EEA1 (*a-c*), anti-CI-M6PR (*d-f*) and anti-ubiquitin (*g-i*), followed by Alexa Fluor 546-conjugated anti-rabbit (*a-f*) and anti-mouse (*g-i*) IgG secondary antibodies. Fluorescence corresponding to CHMP3¹⁻¹⁷⁹GFP is shown in *a*, *d* and *g* (green). Fluorescence corresponding to EEA1, CI-M6PR and ubiquitin is shown in *b*, *e* and *h* respectively (red). Images of merged fluorescence are shown in *c*, *f* and *i* (yellow fluorescence indicates co-localisation). Insets are magnifications of boxed areas. Note: neighbouring untransfected cells do not accumulate ubiquitin (*h*).

Fig. 2 Dominant negative CHMP3 perturbs endo-lysosomal trafficking of EGF. HeLa cells were transfected with appropriate constructs and allowed to express for 24 h until serum-containing medium was removed and replaced with DMEM containing 1% BSA. Cells were serum starved overnight for 16 h and then incubated with 500ng/ml Alexa-Fluor[®] 555-conjugated EGF for 2 min or 60 min at 37°C prior to processing for confocal microscopy. Panels *a-c* show two transfected cells and a single untransfected cell after 2 min of EGF stimulation, with EGF (red) found largely at the plasma membrane of the cells. Panels *d-f* and *g-i* show the results of CHMP3¹⁻¹⁷⁹GFP and CHMP3¹⁻¹⁷⁹GFP^{M1} (green) expression respectively on EGF (red) degradation after a 60 min stimulation. Note the untransfected cells denoted by '*' in panels *d-i*.

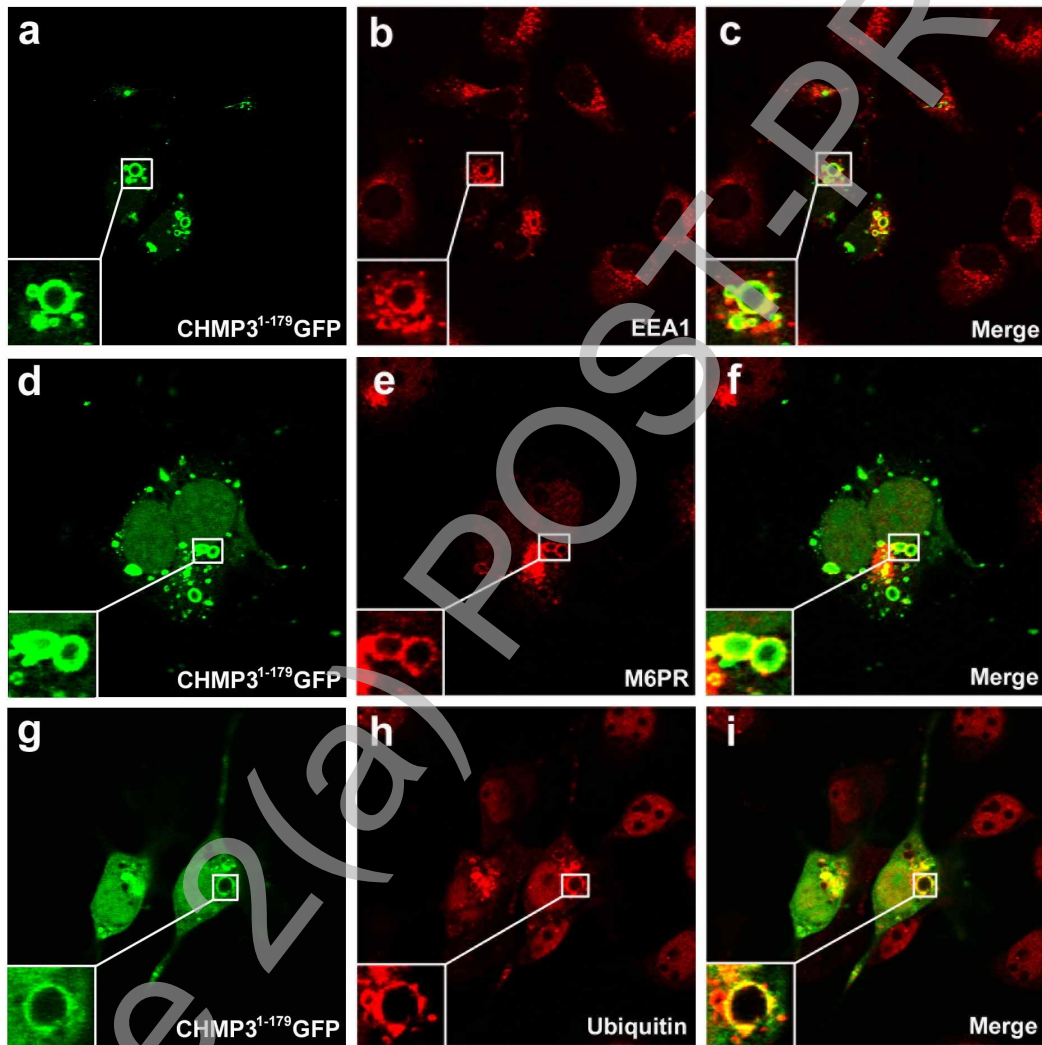
Fig. 3 CHMP3¹⁻¹⁷⁹GFP localises to the mid-body in late stages of cytokinesis. Cos-7 (*a-c*, *g-i*) and HeLa (*d-f*) cells were transfected with CHMP3¹⁻¹⁷⁹GFP as outlined previously. Cells were fixed 24 h post-transfection and immunostained with anti-β-tubulin (*a-f*) and anti-Rab11A (*g-i*) antibodies followed by Alexa Fluor 546-conjugated anti-mouse IgG secondary antibodies. Fluorescence corresponding to CHMP3¹⁻¹⁷⁹GFP is shown in *a*, *d* and *g* (green). Fluorescence corresponding to β-tubulin and Rab11A is shown in *b+e* and *h* respectively (red). Images of merged fluorescence are shown in *c*, *f* and *i*. Insets are magnifications of boxed areas.

Fig. 4 Dominant negative ESCRT component mutants disrupt proper cytokinesis. HeLa cells were seeded on 13 mm coverslips 24 h prior to transfections. Cells were then transiently

transfected with GFP-Vps4^{WT}, GFP-Vps4^{E235Q}, CHMP3¹⁻¹⁷⁹GFP, CHMP3¹⁻¹⁷⁹GFP^{M1} or GFP alone. Cells were fixed 24 h post-transfection and immunostained with anti- β -tubulin followed by Alexa Fluor 546-conjugated IgG secondary antibodies and DAPI. **A.** All transfected cells present on the coverslip were then scored for multinucleation and the results were represented graphically as percentages of multinucleate cells (averaged over 3 experiments; error bars are $\pm SD$, $n=3$). Results were analysed by Chi-squared analysis and dominant negative mutants found to be significantly different to GFP control ($p < 0.001$). Total numbers of cells over 3 separate experiments counted: GFP = 867, GFP-Vps4^{WT} = 1440, GFP-Vps4^{E235Q} = 871, CHMP3¹⁻¹⁷⁹GFP = 1441, CHMP3¹⁻¹⁷⁹GFP^{M1} = 1800. **B.** A field of view at 400x magnification to show cells transfected with CHMP3¹⁻¹⁷⁹GFP and their effects on cytokinesis. Cells denoted by ‘*’ represent a single multinucleated transfected cell. Cells marked as ‘#’ represent a transfected cell that is mononucleate. In this case a multinucleation count would have resulted in 3 positive cells and 1 negative cell for multinucleation.

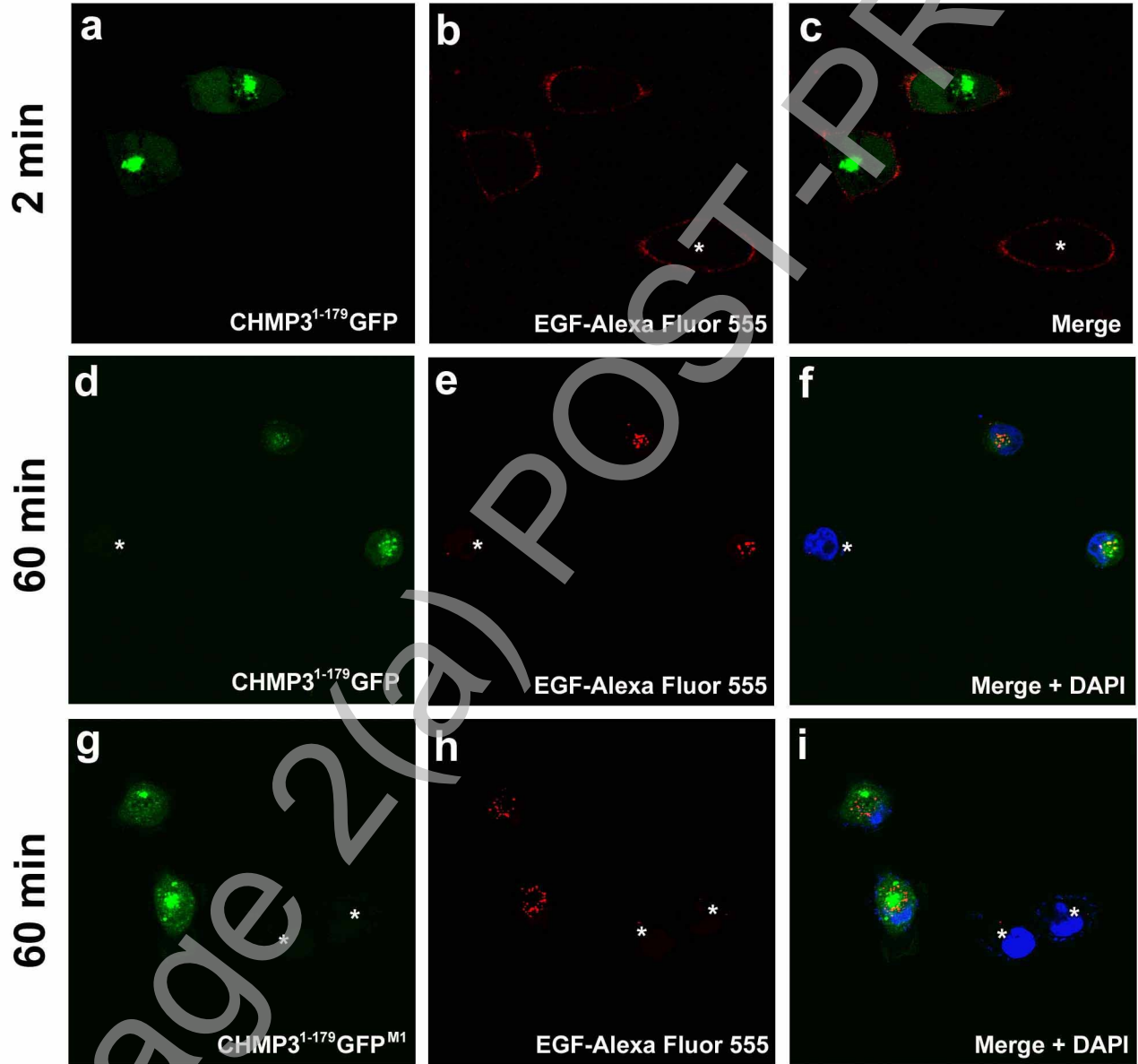
Fig. 5 Expression of mutant CHMP3¹⁻¹⁷⁹-GFP^{M1} results in early and late endosomal localisation as well as disruption of endosomal trafficking and localisation to the mid-body in cytokinesis. Cos-7 (*a-c*) and HeLa (*d*) cells were transfected with the CHMP3¹⁻¹⁷⁹GFP^{M1} fusion construct as outlined previously. Cells were fixed 24 h post-transfection and immunostained for anti-EEA1 (*a*), anti-CI-M6PR (*b*), anti-ubiquitin (*c*) and anti- β -tubulin (*d*) followed by Alexa Fluor 546-conjugated IgG anti-rabbit (*a-b*) and anti-mouse (*c-d*) secondary antibodies. Fluorescence corresponding to CHMP3¹⁻¹⁷⁹GFP^{M1} is shown in *a-d* left-hand insets (green). Fluorescence corresponding to EEA1, CI-M6PR, ubiquitin and β -tubulin is shown in *a-d* right-hand insets respectively (red). Main panel images show merged fluorescence, with yellow indicating co-localisation.

Figure 1



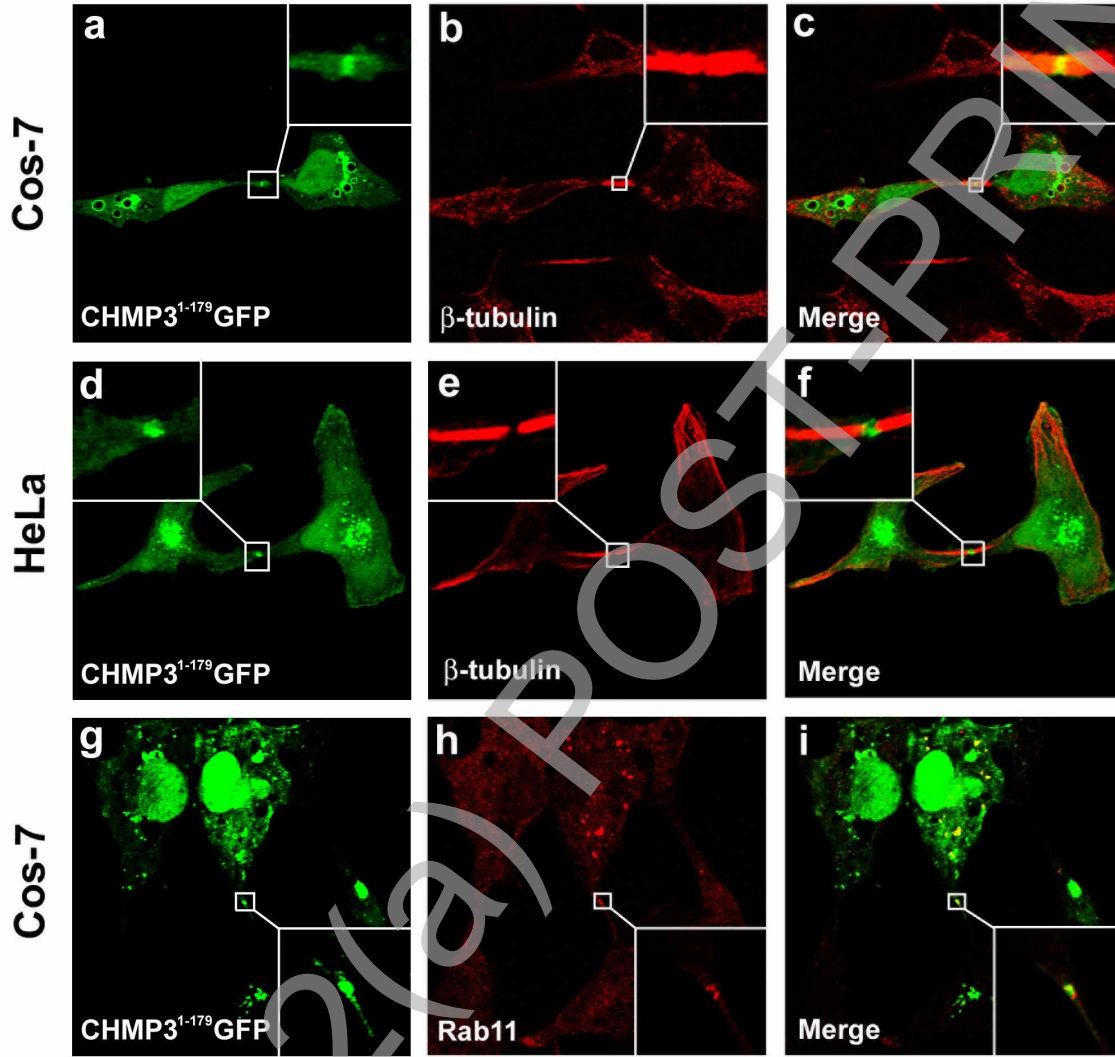
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Figure 2



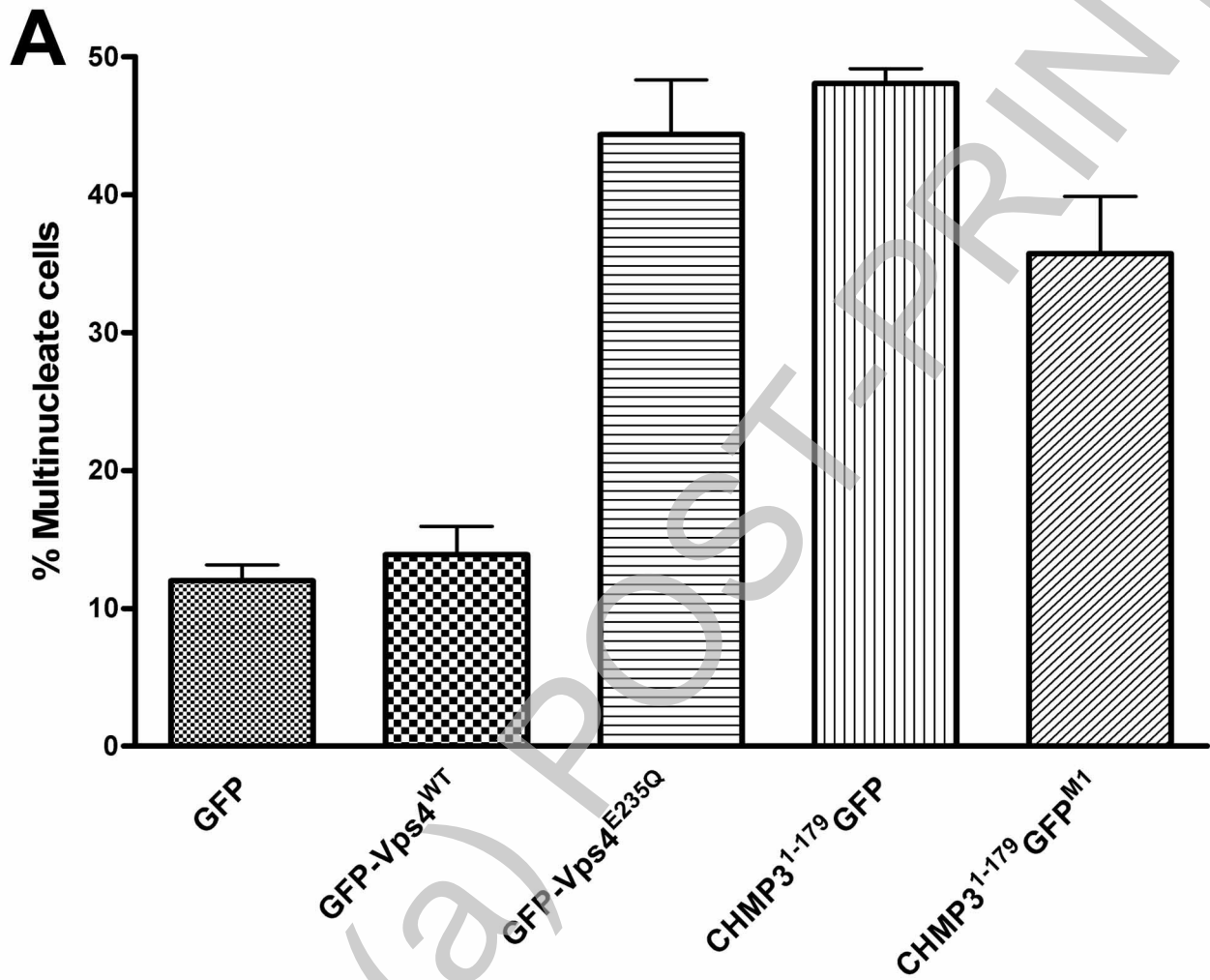
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Figure 3

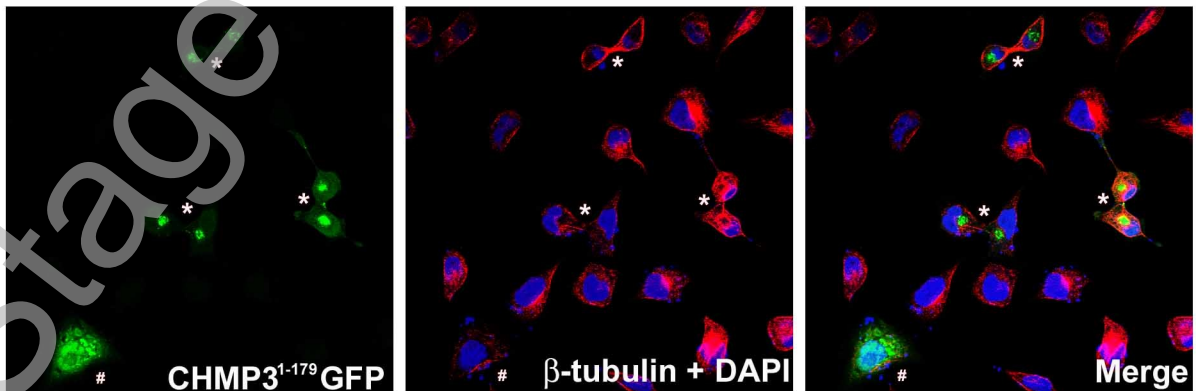


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Figure 4

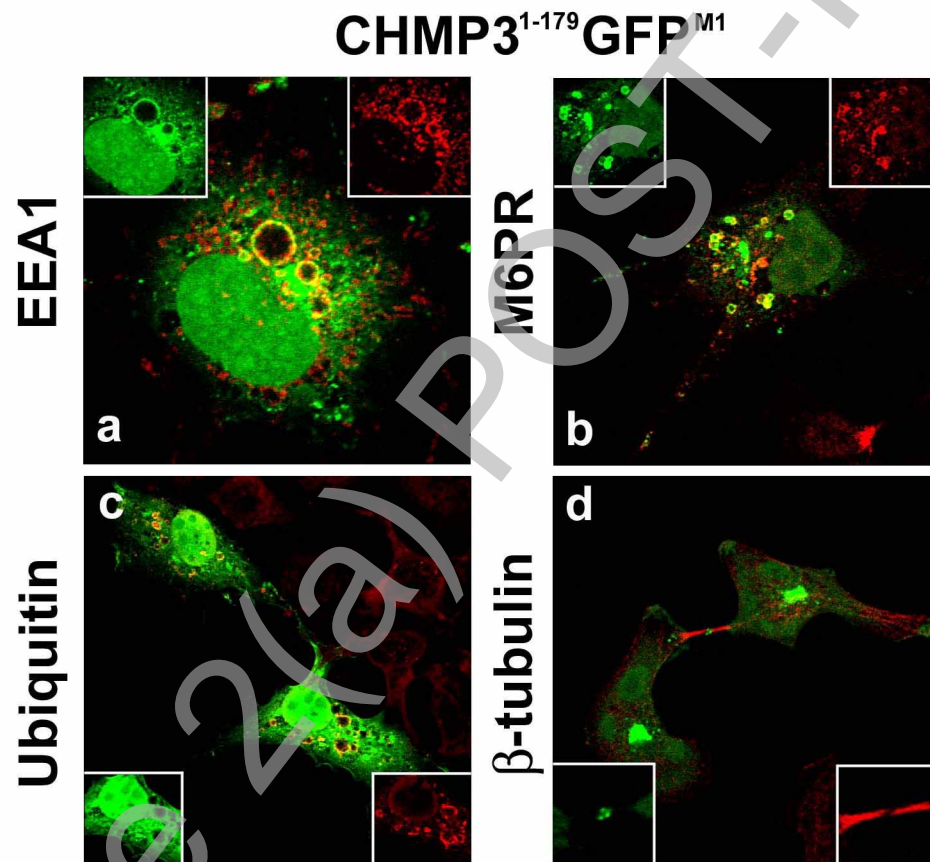


B



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Figure 5



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