REPORT

In situ cryo-electron tomography reveals filamentous actin within the microtubule lumen

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Microtubules and filamentous (F-) actin engage in complex interactions to drive many cellular processes from subcellular organization to cell division and migration. This is thought to be largely controlled by proteins that interface between the two structurally distinct cytoskeletal components. Here, we use cryo-electron tomography to demonstrate that the microtubule lumen can be occupied by extended segments of F-actin in small molecule–induced, microtubule-based, cellular projections. We uncover an unexpected versatility in cytoskeletal form that may prompt a significant development of our current models of cellular architecture and offer a new experimental approach for the in situ study of microtubule structure and contents.

Introduction

Understanding of the lumenal contents of cytoplasmic microtubules has been classically driven by EM-based analysis in cells and tissues (Burton, 1984; Peters and Vaughn, 1967; Rodríguez Echandía et al., 1968), and most recently by high resolution cryo-EM (Atherton et al., 2018; Bouchet-Marquis et al., 2007; Garvalov et al., 2006; Grange et al., 2017; Koning et al., 2008). Contents of cytoplasmic microtubules are thought to be restricted to globular proteins such as tubulin-modifying enzymes (Coombes et al., 2016), although higher order structures have been observed in sperm flagella microtubules and cilia (Ichikawa and Bui, 2018; Zabeo et al., 2018). Unequivocal identification of luminal proteins in a cellular context remains a challenge as the dimensions of the microtubule may confound super-resolution fluorescence imaging techniques, and there are potential issues with antibody-epitope accessibility in this confined environment. Cryo-EM solves the challenge of spatial resolution, but can be limited by the technical requirement for very thin samples (Schur, 2019), so studies have mainly focused on microtubules at the cell periphery or within neuronal processes (Atherton et al., 2018; Bouchet-Marquis et al., 2007; Garvalov et al., 2006; Grange et al., 2017; Koning et al., 2008). We recently identified a compound, 3,5-dibromo-N’-[(2,5-dimethyl-1-(3-nitrophenyl)-1H-pyrrol-3-yl)methylene]-4-hydroxybenzohydrazide, named “kinesor,” that targets the motor protein kinesin-1, promoting extensive remodeling of the microtubule network (Randall et al., 2017). Kinesor treatment results in dynamic looping and bundling of microtubules within the cytoplasm and their extrusion from the cell body as membrane-bound projections. This renders microtubules from within the cell accessible for cryo-EM studies (Cross and Dodding, 2019; Randall et al., 2017).

Here we describe the discovery of filamentous actin (F-actin) inside the microtubule lumen through in situ cryo-electron tomography (cryo-ET) analysis of these small molecule–induced projections.

Results and discussion

Formation and ultrastructure of small molecule–induced, microtubule-based projections

To begin ultrastructural analysis of projections in their native state, HAP1 cells, incubated with a fluorescent membrane stain before kinesor treatment, were prepared for cryo–correlative light EM (CLEM). This allowed the unambiguous identification of projections using fluorescence microscopy that could be correlated with images from the electron microscope (Fig. 1 A). Consistent with our earlier immunofluorescence imaging study (Randall et al., 2017), abundant projections were composed of closely aligned microtubules, and we also occasionally observed vesicular structures within swellings as the periphery. Our previous live-imaging of GFP-tubulin expressing HeLa cells suggested that the projections were initially formed through the extrusion of microtubule loops (Randall et al., 2017). Live-imaging of SiR-tubulin labeled microtubules in the HAPI cells used here confirmed this and showed that microtubules are progressively added through additional loop extrusion events (Fig. S1 and Video 1), providing a rationale for the formation of extended microtubule bundles. Consistent with this live imaging, EM analysis of regions proximal to the cell body revealed a greater number of microtubules and distinct looped bundles (Fig. 1 B), with an internmicrotubule spacing of

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10–25 nm (blue shading), although some were also observed to traverse bundles (yellow shading). These observations are similar to in vitro EM studies of kinesin-mediated microtubule–microtubule cross-linking (≤25 nm spacing; Andrews et al., 1993) and measurements of the distance kinesin-1 holds its cargoes from the microtubule surface (∼17 nm; Kerssemakers et al., 2006).

**Figure 1.** Cryo-CLEM analysis of kinesore-induced projections. HAP1 cells grown on Quantifoil gold EM grids were stained with Cell Mask green to identify plasma membrane and treated with kinesore (100 µM) for 1 h before plunge-freezing and imaging using fluorescence and EM. (A) Low magnification images show fluorescence (green) and EM image (grays) overlay of cells with projections emerging. Regions boxed i and ii are shown at higher magnification and expanded on the right. Black dots are 10 nm gold fiducials. (B) A single EM image acquired proximal to the cell body with a large number of microtubules. Boxes show microtubules with similar alignments (shaded blue, 1–3 [top] and 1–4 [bottom]) and an example of a single microtubule that crosses a bundle of aligned microtubules (yellow, 5 [bottom]). Scale bars are 100 nm.

**Cry-ET reveals actin-like filaments with the lumens of extruded microtubules**

Satisfied that we could confidently identify projections in the electron microscope, additional samples were analyzed without the fluorescence imaging step (avoiding ice contamination) by 3D reconstruction of tomography tilt series (Fig. 2). Transverse
sections through projections revealed that they could contain variable numbers of microtubules (ranging from 4 to >30; Fig. 2 and Fig. S2). A section of a 23-microtubule projection is shown in detail. Microtubules are organized in a twisted bundle, and vesicles are excluded in swellings proximal to the limiting membrane. Both transverse sections and serial images of Z-sections show that the microtubules are intact (Video 2). Surprisingly, within the microtubule lumens, as well as globular structures, we could clearly observe extended filamentous density with helical character (blue arrows). Two examples of such filaments are boxed in orange and blue on the Z-section panels in Fig. 2 and are also visible as density within the microtubule lumen of transverse sections (see also Fig. S2). The size and helical appearance of these lumenal filaments are consistent with those of F-actin (5–9 nm; Chou and Pollard, 2019; Fujii et al., 2010) which, at least in principle, could reside within the ~15–16 nm diameter luminal space (Nogales et al., 1999).

To assess whether kinesore-induced projections do indeed contain actin, methanol-fixed cells (to optimally preserve microtubules) were stained with antibodies against actin and tubulin. This revealed patches and puncta of actin along microtubules within the projections (Fig. 3 A). Actin antibodies may not discriminate between G- and F-actin, so equivalent samples were prepared using PFA fixation and phalloidin staining for F-actin. Under these fixation conditions, projections were less well preserved, with fragmentation in β-tubulin staining, but actin patches were more prominent and appeared to bridge gaps in tubulin staining (Fig. 3 B). This raises the
intriguing possibility that a population of F-actin may reside within the microtubule lumen that is refractory to traditional means of detection, possibly due to limited antibody epitope/phalloidin binding site accessibility or conformation. Supporting this proposition, a brief (60 s) cold treatment that disrupted tubulin staining enhanced actin antibody staining to the extent that actin staining defined the projection structure (Fig. 3 C).
Lumenal actin-like filaments have two distinct morphologies
A survey of the gross morphology of lumenal filaments resulted in their classification into two pools: Class I and Class II. Class I filaments are exemplified in the right orange box in Fig. 2 A, and Z-sections and class II by the left blue box. Further examples are shown in Fig. 4 A. Video 3 shows a Z-series through a microtubule containing an extended Class I filament adjacent to an “empty” microtubule. Class II filaments appeared slightly thicker and were typically better defined than Class I. Analysis of the tomograms in our dataset that provided clearest definition of lumenal contents (n = 11), which contained 146 microtubules with a total length of 113 µm, revealed that 27% of the total lumenal length of microtubule was occupied by actin-like filaments of either class (Fig. 4 B). There was considerable variation between tomograms with 4–76% of the lumenal length occupied, with a mean of 29%. Of the total actin-like filament length, 68% were Class I, and 32% were Class II filaments (Fig. 4 C). Although the frequencies of Class I and Class II filaments were similar, Class I filaments were longer (average, 475 nm) than Class II (average, 274 nm; Fig. 4, D and E). Microtubules containing Class I filaments had average outer and lumenal diameters of 25.56 ± 0.59 nm and 16.84 ± 0.76 nm, respectively. Those containing Class II filaments were typically slightly wider at 27.29 ± 0.58 nm (outer diameter) and 17.80 ± 0.85 nm (lumenal diameter), indicating that the presence of lumenal filaments either correlates with or modifies microtubule properties (Table S1). The measured Class I diameters are close to those of a 13 protofilament microtubule (Zhang et al., 2018), which may suggest that Class II microtubules have an expansion in the lattice, or perhaps, more protofilaments.

Layer-line analysis confirms that lumenal filaments are composed of F-actin
To further characterize microtubule cores, lumenal regions were extracted from the tomographic reconstructions as 3D sub-volumes. These were then summed in Z to obtain 2D projection images. Fourier transforms of these images were then calculated and inspected. Helical filaments like actin and tubulin display distinct layer-line patterns. Data from single representative microtubules containing filaments of the Class I and Class II varieties are shown in Fig. 5, and measured parameters from several microtubules/filaments are shown in Table S1. The layer lines we observe are highly consistent with prior analysis of the helical parameters of actin filaments extracted from tomograms of mammalian cells (Narita et al., 2012). Class I filaments display an actin layer-line pattern: a clear reflection at 5.94 ± 0.03 nm, the pitch of the short left-handed actin genetic helix (Actin Turn [L]), and a reflection at 29.53 ± 0.81 nm corresponding to the crossover spacing of the two right-handed long pitch actin helices (Actin Cross). This crossover spacing is shorter than for canonical actin (~35 nm) but greater than that reported for actin cofilin filaments (~27 nm; Hanson, 1967; McGough et al., 1997).

Figure 4. Abundance and classification of two types of actin-like filament. (A) Examples of lumenal filaments extracted from sub-volumes of 3D tomograms and projected in Z, chosen from straight sections of microtubule so that lumenal filaments can be shown over an extended length without being obscured by surrounding microtubule lattice. Images show projected sections of central regions of microtubules containing actin-like filaments and are separated into two groups (Class I and Class II) based on their morphology. Class II filaments were thicker and typically better defined. Scale bar is 25 nm. (B) Graph shows the proportion of microtubule lumen occupied by actin-like filaments (of either class) in 11 tomograms. (C) Proportion of actin-like filament lengths classified as Class I or Class II from the dataset. (D and E) Graph showing the average length of actin-like filaments (error bars represent SEM) of either class and their frequency distribution.
and actin is known to have a variable twist (Egelman et al., 1982). These results enable us to confidently identify the Class I lumenal filament as F-actin. Increased twist when compared with canonical actin may partly explain why phalloidin staining of this actin pool appears restricted and likely has implications for its detection with other probes (Kumari et al., 2019; McGough et al., 1997). A reflection at 4 nm was also visible as expected from the tubulin monomer axial repeat. Class II filaments also gave an actin layer-line pattern with reflections at 6.11 ± 0.09 nm (Actin Turn [L]) and 27.44 ± 2.28 nm (Actin Cross) that is augmented by a strong meridional reflection at 6.18 ± 0.06 nm (errors are SD). SEM is provided in Table S1 with measurements of additional actin reflection (Actin Turn [R]) at the pitch of the right-hand genetic helix. (B) 3D maps from representative extracted filament volumes using real space helical reconstructions with helical parameters derived from the models presented in Fig. S3 and Table S1. Maps are low pass filtered to 30 Å.

In summary, we have shown that microtubules can contain actin filaments within their lumen. We call these structures microtubule lumenal actin. We have distinguished two types of structure, Class I and Class II, with slightly different actin helical symmetries and outer microtubule diameters. The discovery of microtubule lumenal actin in our cell-based extrusion system prompts the questions of how it is incorporated, its functions, and its abundance in settings that are not small molecule–modified. Two hypotheses present themselves that are not mutually exclusive. First, altered microtubule dynamics caused by activation of kinesin-driven microtubule–microtubule sliding and bundling may provide an opportunity for actin to enter the microtubule lumen. This may occur through transient opening of the microtubule under strain when tight loops are formed (Fig. S1) or by kinesin-mediated disruption of the lattice (Coombes et al., 2016; Schaedel et al., 2019; Triclin et al., 2018). Put another way, F-actin incorporation may occur in response to mechanical or structural stress (Brangwynne et al., 2006), so a potential role for microtubule severing enzymes should also be considered (Vemu et al., 2018). Alternatively, microtubule extrusion has facilitated the analysis of a pool of preexisting actin-containing microtubules with previously limited accessibility by...
Materials and methods

Cell culture and small molecule treatment
HAPI cells were obtained from Horizon Discovery and cultured in Iscove’s Modified Dulbecco’s Medium containing 10% FCS and penicillin/streptomycin at 37°C in a 5% CO₂ incubator. For fluorescence imaging, cells were plated onto fibronectin-coated coverslips in a six-well plate at a density of 10⁵ per well the day before small molecule treatment. Cells were prepared for EM as described below. Kinesin (3,5-dibromo-N’-[(2,5-dimethyl-1-(3-nitrophenyl)-1H-pyrrol-3-yl)methylene]-4-hydroxy benzohydrazide) was obtained from Chembridge Corporation (6233307) and prepared as a 50 mM stock in DMSO. In addition to manufacturer-provided quality control, molecular weight was verified by mass spectrometry. To stimulate projection formation, treatments were performed in Ringer +20° to +60° with angular increments of 3°, total dose of 109 e/Å², and a defocus range between −2 μm and −4 μm. The acquisition magnification was 63,000 times, resulting in calibrated pixel size of 2.21 Å. Images were recorded in counted mode using a K2 Summit direct electron detector (Gatan) fitted to a BioQuantum energy loss spectrometer (Gatan) operating with a 20-eV slit width.

Data processing
Reconstructions were performed using IMOD and its EtoMO interface (University of Colorado, Boulder; Kremer et al., 1996). Briefly, image stacks containing bidirectional tilt-series data were imported into the software and subjected to coarse alignment using Tiltxcorr before fine fiducial-based alignment, positioning, and 2 × 2 binning. Tomograms were generated using the Weighted Back Projection with the Simultaneous Iterative Reconstruction Technique like filter to five iterations. The nominal voxel size of the final tomograms was 4.414 Å. Further processing and analysis were performed using the IMOD3dmod interface to generate videos, extract sub-volumes, and perform microtubule/actin filament length analysis. Microtubules were modeled using 24-nm-diameter cylinder segments. The outer and lumenal diameters of the microtubules were measured on 2D projection images of extracted sub-volumes using Fiji. Images and sub-volumes were exported to Fiji or Adobe Photoshop to prepare for publication or further analysis as described below.

Layer-line analysis
Fourier transforms of 2D projections of extracted filament volumes were calculated and the layer-line positions measured using Fiji (ImageJ). The well-characterized 4 nm reflection of the tubulin monomer was used as an internal calibration tool and the real space pixel size for each filament calculated. Modeling of...
the layer-line patterns from helices with actin-like symmetry (Fig. S3) was performed using the HELIX program (Knupp and Squire, 2004; https://www.diamond.ac.uk/Instruments/Soft-Condensed-Matter/small-angle/SAXS-Software/CCP13/HELIX.html). A HELIX plug-in for ImageJ (https://imagej.nih.ij) can be obtained from c.knupp@gmail.com.

Helical reconstruction

To directly compare the 3D structures of the two filament classes, real space helical reconstructions of individual filaments were calculated using EMAN2 (Tang et al., 2007). 85 nm long overlapping segments were extracted from 2D projections of representative filaments. The axial rise and subunit rotation determined from the modeling experiments and weighted back projection were used for the reconstruction. These representative 3D volumes were low-pass-filtered to 30 Å to minimize the high-frequency noise present in single-filament images.

Online supplemental material

Fig. S1 shows live imaging of projection formation. Fig. S2 shows high-frequency noise present in single-filament images. Fig. S3 shows layer-line analysis and measurements of microtubule dimensions. Video 1 shows HAP1 cells labeled with SiR-tubulin and treated with kinesore. Video 2 shows a series of images through the tomogram described in detail in Fig. 2. Video 3 shows Z-series through a sub-volume containing two microtubules, one with a luminal actin filament.

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Figure S1.  **Live-cell confocal imaging of microtubule projection formation through loop-extrusion in SiR-tubulin–labeled HAP1 cells.**  

(A) Left image shows cells at T = 0 s. Right image shows T = 0, T = 585, and T = 1,200 (time in s) in red, green, and blue channels, respectively. Boxes highlight areas that are expanded and analyzed in B and C. Scale bar, 10 µm.  

(B) Stills from the image series describing the formation and reinforcement of a microtubule-based projection through the extension/resolution of a loop. Green panels show extension of the first loop (event 1). Top graphs show change in tubulin integrated (Int.) intensity over the whole region as a function of time. The lower graph shows the average tubulin intensity per line as a function of distance along the axis of projection extension (x axis) and rate of extension. Magenta panels (event 2) show addition of further tubulin intensity over a similar time period later in the video, indicating that the projections are formed by the progressive layering of microtubules from the extrusion of loops.  

(C) Complex interactions between projections that affect their organization. Three extensions are highlighted by light blue, light green, and magenta dotted lines. The magenta projection moves along the light blue projection. The position of the light green filament is shifted as it passes. Green box panel shows original (dark green) and new positions (light green) of the projection. As the magenta proceeds along the blue filament, the blue projection is bent. Blue boxed panel shows original (dark blue) and new positions (light blue) of the blue projection.
Figure S2. **Sections through microtubule-based projections reveal variable microtubule number and close-packed organization.** Transverse sections through several microtubule-based projections show the close juxtaposition of variable numbers of microtubules. Lumenal density in many of the microtubules is apparent. Images are presented as summed slices. Scale bars, 100 nm.
Figure S3. **Modeling and diffraction of actin filaments.** (A) Comparison of the radial nets of muscle actin filaments (13/6 helices of actin monomers) and Class I and Class II actin filaments as observed here, where Class I filaments have approximate 11/5 helical symmetry and Class II filaments have 20/9 helical symmetry. The inset table shows the helical parameters for the three symmetries. (B) Diffraction from Class I and Class II filaments and comparison to models. The images on the left reproduce the patterns shown in the main text Fig. 5 for Class I (top) and Class II (bottom) filaments. On the right are patterns produced by the HELIX program (Knupp and Squire, 2004) for actin-like filaments with 11/5 (top) and 20/9 (bottom) helical symmetry. A filament length of 100 subunits (simple spheres) was used with the monomer at 25 Å from the filament axis.
Video 1. **Live-imaging of tubulin in kinesore-treated HAP1 cells.** Video shows HAP1 cells labeled with SiR-tubulin and treated with kinesore that is also shown in Fig. S1. Images were acquired every 15 s. Boxed region shows area of video described in more detail in Fig. 1.

Video 2. **Tomographic analysis of microtubules within a projection.** Video shows a series of images through the tomogram described in detail in Fig. 1 B. Microtubules in the tomogram are then identified by colored cylinders.

Video 3. **Z-series through a Class I filament-containing microtubule.** Video shows Z-series through a subvolume containing two microtubules. The top microtubule contains a Class I filament. The bottom microtubule contains globular densities.

Table S1 is provided online as a separate Excel file. Table S1 shows measured parameters for individual Class I and Class II filaments. Microtubule dimensions and layer-line positions are listed for filaments of both classes. All data are shown in Angstroms. A comparison of observed (OBS) vs. calculated (CALC, from models) layer-line positions is also presented. N/A, not applicable as meridional layer line is not present in class I filaments.