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Nucleation and capture of large cell surface-associated microtubule arrays that are not located near centrosomes in certain cochlear epithelial cells

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ABSTRACT

This report deals with the as yet undetermined issue of whether cell-surface associated microtubules in certain cochlear epithelial cells are centrosomally nucleated and subsequently migrate to microtubule-capturing sites located at the surface regions in question. Alternatively, the cells may possess additional nucleating sites which are noncentrosomal and surface-associated. These alternative possibilities have been investigated for highly polarised epithelial cells called supporting cells in the mouse and guinea pig organ of Corti using antibodies to pericentrin and γ-tubulin. There is substantial evidence that both proteins are essential components of microtubule-nucleating sites in cells generally. Each mature supporting cell possesses a large microtubule array that is remotely located with respect to its centrosome (more than 10 μm away). The antibodies bind to a cell's centrosome. No binding has been detected at 2 other microtubule-organising centres that are associated with the ends of the centrosomally-remote microtubule array while it is being constructed. Such arrays include thousands of microtubules in some of the cell types that have been examined. If all a cell's microtubules are nucleated by its centrosome then the findings reported above imply that microtubules escape from the centrosomal nucleating site and migrate to a new location. Furthermore, capture of the plus *and minus* ends of the errant microtubules is taking place because both ends of a centrosomally-remote microtubule array are attached to sites that are precisely positioned at certain cell surface locations. Minus ends are locating targets with an exactitude comparable to that which has been demonstrated for plus ends in certain cell types. These cells apparently operate a single control centre strategy for microtubule nucleation that is complemented by precise positioning of plus and minus end-capturing sites at the cell surface.

Key words: Organ of Corti; cytoskeleton; centriole; γ-tubulin; pericentrin.

INTRODUCTION

Certain epithelial cells called supporting cells collectively provide a highly ordered cytoskeletal framework in the mammalian organ of Corti (see Henderson et al. 1995). Exact spatial organisation of this framework is functionally important because it connects sensory hair cells to the basilar membrane which vibrates during hearing. Both ends of certain microtubule bundles are very precisely positioned at particular surface sites in supporting cells but they are not located near centrosomal microtubule-organising centres (Henderson et al. 1995; Tucker et al. 1995) although the centrosome is the main microtubule-nucleating site in most animal cell types (see Kalnins, 1992; Kimble & Kuriyama, 1992; Kalt & Schliwa, 1993; Kellogg et al. 1994). This report is concerned with the question of whether supporting cells possess additional (noncentrosomal) cell surface-associated microtubule-nucleating sites to assist in spatial control

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of microtubule positioning. Antibodies to pericentrin and γ-tubulin have been used.

Pericentrin and γ-tubulin are included in the pericentriolar material of centrosomes and these 2 proteins are also present at other (noncentrosomal) nucleating sites which lack centrioles in a wide range of other cell types (e.g. Gueth-Hallonet et al. 1993; Liu et al. 1993; Doxsey et al. 1994; Horio & Oakley, 1994; Debec et al. 1995). Pericentrin and γ-tubulin are essential components of microtubule-nucleating sites that have been investigated because they participate in the molecular interactions involved during microtubule nucleation to form the minus ends of microtubules (see Doxsey et al. 1994; Joshi, 1994; Stearns & Kirschner, 1994; Moritz et al. 1995; Oakley, 1995; Zheng et al. 1995). After nucleation, outgrowth of microtubules from a nucleating site such as a centrosome involves polarised elongation which progresses by further addition of microtubule proteins to the other (plus) ends of the microtubules. In some cases, the dynamically unstable plus ends encounter capturing sites where they are capped and stabilised (see Kirschner & Schulze, 1986; Gelfand & Bershadsky, 1991).

Some cells possess microtubules which are distantly located with respect to their centrosomes. There is substantial evidence that in neurons such microtubules are released from centrosomes after nucleation for translocation to their final destinations (see Joshi & Baas, 1993; Ahmad & Baas, 1995). There is evidence for release of centrosomally nucleated microtubules in other cells (Vorobjev & Chentsov, 1983; Belmont et al. 1990; McBeath & Fujiwara, 1990; Henderson et al. 1994, 1995) and it has recently been established that this occurs in certain cultured epithelial cells (Keating et al. 1997). In several epithelial cell types the minus ends of microtubules are closely associated with the apical cell surface (Bacallao et al. 1989; Meads & Schroer, 1995; Mogensen et al. 1989); most microtubules do not project from the centrosome although it is situated apically. In certain epithelial cells the situation is more pronounced. The minus ends of many of their microtubules are associated with sites at the cell surface but their centrosomes are not close to the surface regions in question (Troutt & Burnside, 1988; Henderson et al. 1994, 1995; Vogl et al. 1995). Examination of microtubule reassembly after exposure to nocodazole has provided evidence for nucleation at surface sites which are remotely located with respect to centrosomes in Sertoli cells (Vogl et al. 1995) but a surface capture strategy apparently operates after microtubules have undertaken long range migrations from the centrosomal region in certain cochlear supporting cells (Henderson et al. 1994, 1995).

Each supporting cell constructs 2 microtubule arrays and possesses 3 main microtubule-organising centres (MTOCs) where nucleation or capture of microtubules occurs. One of the arrays elongates from an apical cell surface-associated region where the centrosome is situated but much of the construction of the other array proceeds subsequently in the lower portion of a cell at a location which is remote (more than 10 μm away) with respect to the centrosome. This investigation deals with the localisation of pericentrin and γ-tubulin during the assembly and positioning of the microtubule arrays which are remotely located with respect to a centrosome when their construction is complete. Are pericentrin and γ-tubulin present at the cell surface sites where the ends of these arrays are located while assembly and positioning of the arrays is proceeding? The arrays are much larger than any described in other mammalian cell types; they have lengths of up to 40 μm and include up to 4500 microtubules (Tucker et al. 1992, 1995; Henderson et al. 1994, 1995). Hence, cochlear supporting cells provide substantial and well separated targets for analyses which seek to identify the location of sites where microtubule nucleation and capture occur. They are particularly favourable material for exploring the spatial and functional interrelationships between centrosomes and other MTOCs during the assembly of microtubule arrays.

A recent investigation of supporting cells was confined to events that occur when microtubule assembly begins at cell apices. It provided evidence that microtubules which are attached to apical cell surfaces have migrated from nearby centrosomes where their nucleation is effected (Mogensen et al. 1997). This study deals with the construction of microtubule arrays that subseqently proceeds at lower levels in the cells. Is precise positioning of these arrays achieved by capture of both plus and minus ends of their microtubules at cell surface-associated sites after long range migration from centrosomes? Are *all* the cells' microtubules centrosomally nucleated?

MATERIALS AND METHODS

Antibodies

The cDNA encoding pericentrin was isolated from a mouse lambda gt 11 expression library using anti-centrosome autoimmune antisera from scleroderma patients. Fusion proteins produced from 3 different nonoverlapping regions of the pericentrin cDNA were

used to generate rabbit polyclonal antisera which were subsequently affinity purified (Doxsey et al. 1994).

Two anti-γ-tubulin antisera have been used. Both were produced in rabbits and were affinity purified. One was raised against a peptide from the C-terminus of *Xenopus* γ-tubulin (Stearns et al. 1991; Stearns & Kirschner, 1994), the other (serum R75) was raised against a peptide from the C-terminus of human γ-tubulin (Lajoie-Mazenc et al. 1994). Fluorescein-conjugated goat antirabbit IgG (H + L) (Jackson ImmunoResearch Laboratories, Stratech Scientific Ltd., Luton, UK) was used as secondary antibody.

Monoclonal anti-α-tubulin (Amersham International, Aylesbury, UK) was used in conjunction with Texas Red-conjugated goat antimouse IgG (H + L) (Jackson ImmunoResearch Laboratories) as secondary antibody.

Immunolabelling of cryostat sections

Cochleas were isolated from mice (Swiss CD1) by dissection (Tucker et al. 1992) on d 3, 7, and 9 after birth (the period 0–24 h after birth is d 0). They were immersed in Tissue Tek (optimum temperature embedding medium, Leica, Milton Keynes, UK) for 5 min, transferred to a precooled (−20 °C) chuck and immersed for 2 min in 2-methyl butane (isopentane) at −130 °C in a bath of liquid nitrogen. Sections (6–10 μm thick) were cut at −22 °C with a Leica 2800 E Frigocut, transferred to 'polysine' slides (Merck/BDH UK, Poole, UK) and air dried at room temperature. The sections were fixed with absolute methanol at −20 °C for 5 min (followed by absolute acetone at −20 °C for 5 min when antibodies to pericentrin were used). Sections were rehydrated in PBS containing 0.05% Triton X-100 for 15 min.

Some of the cochleas destined for antipericentrin labelling were fixed in 4% paraformaldehyde in PBS buffer at pH 7.2 for 30 min and immersed in Tissue Tek for 15 min. After freezing, sectioning and collection on slides as described above (but without air drying and further fixation), sections were immediately immersed in PBS containing 1% goat serum (Sigma, Poole, UK).

Sections obtained by both the above procedures were blocked in 10% goat serum in PBS for 1 h at room temperature and incubated in a moist chamber with antipericentrin antiserum (diluted 1:300 in PBS with 1% goat serum) or anti-γ-tubulin antiserum (diluted 1:1000 in PBS with 1% goat serum; 1:5 for immunoaffinity purified anti-γ-tubulin IgG from serum R75) for 18 h at 4 °C. After a wash in PBS with 0.05% Triton X-100 for 15 min and 2 washes (30 min each) in PBS with 1% goat serum the sections were blocked with 10% goat serum in PBS for 1 h, incubated with secondary antibody (diluted 1:400 in PBS with 1% goat serum) for 30 min, washed twice for 30 min in PBS, and once for 15 min in distilled water. Sections were mounted in 2.5% N-propyl gallate dissolved in glycerol diluted to 90% with PBS and the edges of coverslips were sealed with dental wax.

Immunolabelling of whole mounts of the organ of Corti

Mature guinea pigs (Dunkin Hartley strain) were killed by cervical dislocation. The bullae were removed and immersed in Hepes buffered (10 mM, pH 7.2) Hanks balanced salt solution (Gibco BRL, Paisley, UK). The ossified capsule surrounding each cochlea was prised away with forceps; this operation also removed the stria vascularis but left the organ of Corti attached to the central bony core. Cochlear cores were fixed in absolute methanol at −20 °C for 3 min. Two washes (each for 2 min) in PBS with 1% goat serum were followed by blocking in 10% goat serum in PBS for 1 h. Incubation with primary antibodies proceeded for 18 h at 4 °C. Primary antibodies were used at the following concentrations: antisera to γ-tubulin and pericentrin were diluted 1:1000 in PBS with 1% goat serum, and the antiserum to α-tubulin was diluted 1:200 in PBS with 1% goat serum. After 6 washes in PBS (10 min each) with 1% goat serum, cochlear cores were immersed in a blocking serum (10% goat serum in PBS) for 30 min and at this point the organ of Corti was dissected away from each core using iris forceps and a fine needle. The helical strip-shaped organs were cut into portions with lengths of 1–2 mm. Organ portions were incubated with secondary antibodies (diluted 1:400 in PBS with 1% goat serum). Six 10 min washes in PBS were followed by 1 in distilled water for 5 min. The specimens were mounted on slides as described above for sections.

Microscopy

Fluorescent images were recorded with a Bio-Rad (Hemel Hempsted, UK) MRC 600 Series laser scanning/confocal imaging system operated in conjunction with a Nikon (Kingston, UK) Diaphot inverted microscope. Images were collected with a ×60 planapo phase contrast objective (N.A. 1.4) and by averaging 5 scans at each focal level using a Kalman filter. Superimposition of a fluorescent image (obtained by confocal scanning) and a phase contrast

image of the same region (acquired by nonconfocal laser scanning) was effected by using one of the two image collection channels in conjunction with a transmitted light detector and employing Bio-Rad 600 software to merge images. Micrographs were obtained using a UP 5000P (Sony UK, Staines, UK) video-printer.

Organs were prepared for electron microscopy using previously described procedures (Tucker et al. 1992; Henderson et al. 1994).

Control procedures

Specificity of antibody binding was assessed by monitoring the fluorescence of organ preparations: after omitting primary antisera, after using pre-immune sera substituted as the source of primary antibodies, and after antibodies had been pre-incubated with the immunising peptides (see Results for further details).

RESULTS

Microtubule-organising centres and microtubule arrays

The organ of Corti consists mainly of a strip of neuroepithelial tissue. Supporting cells and sensory hair cells are arranged in rows parallel to the longitudinal axis of this strip. The strip has an inner and an outer side which are related to its position in the cochlea (see Lim, 1986). A row of inner hair cells is separated from 3 rows of outer hair cells by a row of supporting cells (inner pillar cells). Four rows of supporting cells (a row of outer pillar cells and 3 rows of Deiters cells) run alongside the 3 rows of outer hair cells and their apices interdigitate between those of the hair cells to varying degrees depending on the supporting cell type in question (Figs 1, 2). The bases of all the supporting cells are situated on a specialised basement membrane, the basilar membrane. A continuum of large intercellular spaces lie between the lateral surfaces of most cells and their neighbours along much of their lengths (Fig. 1).

Supporting cells and hair cells possess apical centrosomes (Fig. 1) which each include 2 adjacent centrioles (Fig. 3). All supporting cell types mentioned above construct 2 large microtubule arrays. One of the arrays projects from the apical surface where the centrosome is situated but the other is remotely located with respect to the centrosome (at least 10 μm away at its nearest point, Fig. 1); these arrays will be referred to as the centrosomal and remote microtubule arrays, respectively. Furthermore, remote arrays include substantially more microtubules than the centrosomal arrays (at least twice as many) (compare Figs 4, 5). Values for remote and centrosomal arrays are about: 2000 and 1000 for inner pillar cells (Henderson et al. 1994), 4500 and 1300 for outer

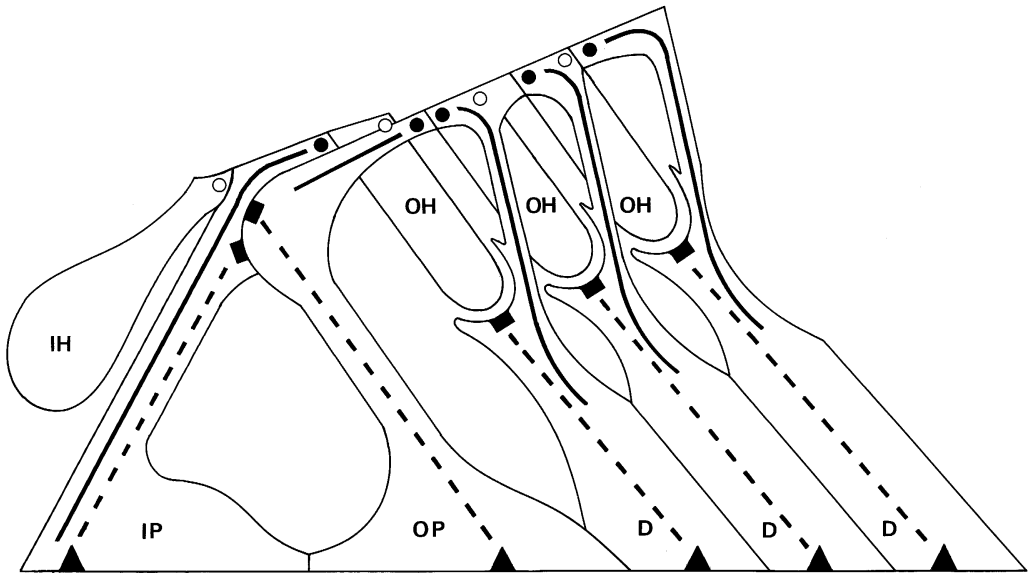

Fig. 1. Schematic diagram of the sensory region of a mature organ of Corti of a mouse or guinea pig cut at right angles to the rows of inner hair (IH), inner pillar (IP), outer pillar (OP), outer hair (OH) and Deiters (D) cells. The apical surfaces of cells are oriented towards the top of the diagram; the outer sides of cells face towards the right. The thick black line inside each supporting cell shows the location of its centrosomally associated microtubule array; the thick broken line shows the position of the array that is remotely located with respect to the centrosome. The positions of centrosomes (●) and noncentrosomal cell surface-associated microtubule-organising centres of supporting cells (▲, ■) are also shown. The centrosomes of hair cells (○) are shown superimposed on the profiles of adjacent supporting cells because of the ways in which cell apices interdigitate (see Fig. 2). Bar, 10 μm.

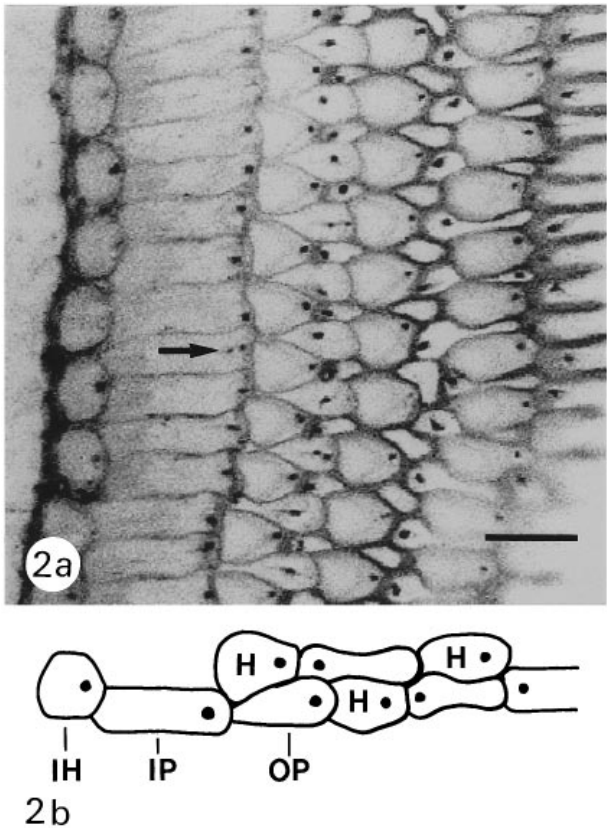

Fig. 2. (*a*) Optical section through the apical portion of a mature guinea pig organ of Corti in the region depicted in Fig. 1. The organ was incubated with a high concentration of an antiserum containing antipericentrin (which had not been affinity purified) and a complementary fluorescein-conjugated secondary antibody. Contrast reversal has been used; the darkest regions are those which fluoresce most intensely. Staining of cell boundaries has occurred. In most cells a single punctate site has also stained in the region where the centrosome is located. In a few cases, staining is concentrated at 2 discrete points at each of these sites (arrow). Confocal laser scanning microscopy. (*b*) Drawing showing the shapes and positioning of cell apices and centrosomal locations to assist identification of the cell types shown in a: inner hair (IH), inner pillar (IP), outer pillar (OP), and outer hair (H) cells. It is only in Deiters cells (unlabelled) that centrosomes are located near the inner sides of cell apices (towards the left of the figure). Bar, 10 μm.

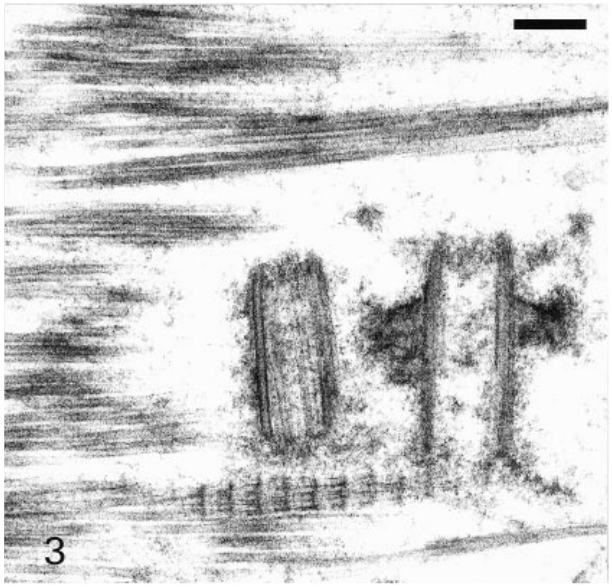

Fig. 3. Section through the centrosomal centrioles and part of the centrosomally associated microtubule array in a mature guinea pig outer pillar cell. Bar, 0.2 μm.

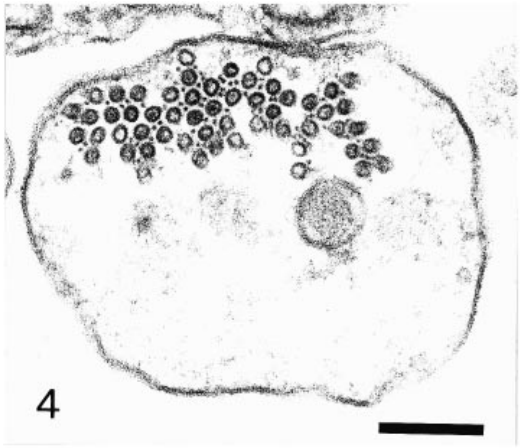

Fig. 4. Cross-section of the microtubule array that projects down from the apical centrosome in a Deiters cell and along a slender cell extension (see Fig. 1) to the main cell body. Mouse, d 21. Bar, 0.2 μm.

pillar cells (Tucker et al. 1995), and 800 and 50 for Deiters cells, respectively.

Each cell possesses 3 cell surface-associated microtubule-organising centres where the ends of microtubule bundles are positioned close to the cell surface (Fig. 1) and the ends of microtubules are embedded in compact meshworks of fibrous material (Fig. 6) (Henderson et al. 1995; Tucker et al. 1995).

Distribution of pericentrin and γ-tubulin during microtubule nucleation

Assembly of microtubule arrays in mouse supporting cells is a postnatal event. Previous studies have shown that microtubules have started to assemble in the apical centrosomal region of inner pillar cells and outer pillar cells by d 1 and 3 after birth, respectively. Early stages in the construction of the remote arrays were detectable by d 6. Microtubule nucleation was definitely taking place in the centrosomal region of inner pillar cells on d 1 and 2, and on d 3–6 in outer pillar cells. Assembly of microtubule arrays was still progressing in both types of pillar cells on d 9 in terms of microtubule elongation (Tucker et al. 1992, 1995; Henderson et al. 1995). Electron microscope examination of section sequences cut through the organs of young mice revealed that microtubules were assemb-

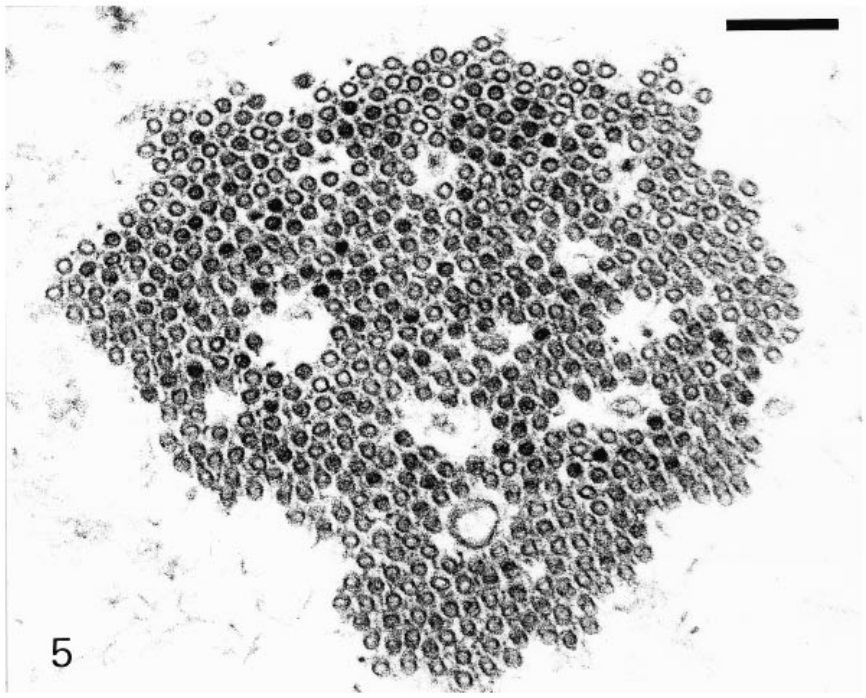

Fig. 5. Cross section through a Deiters cell's microtubule array which is remotely located with respect to the centrosome. Mouse, d 21. Bar, 0.2 μm.

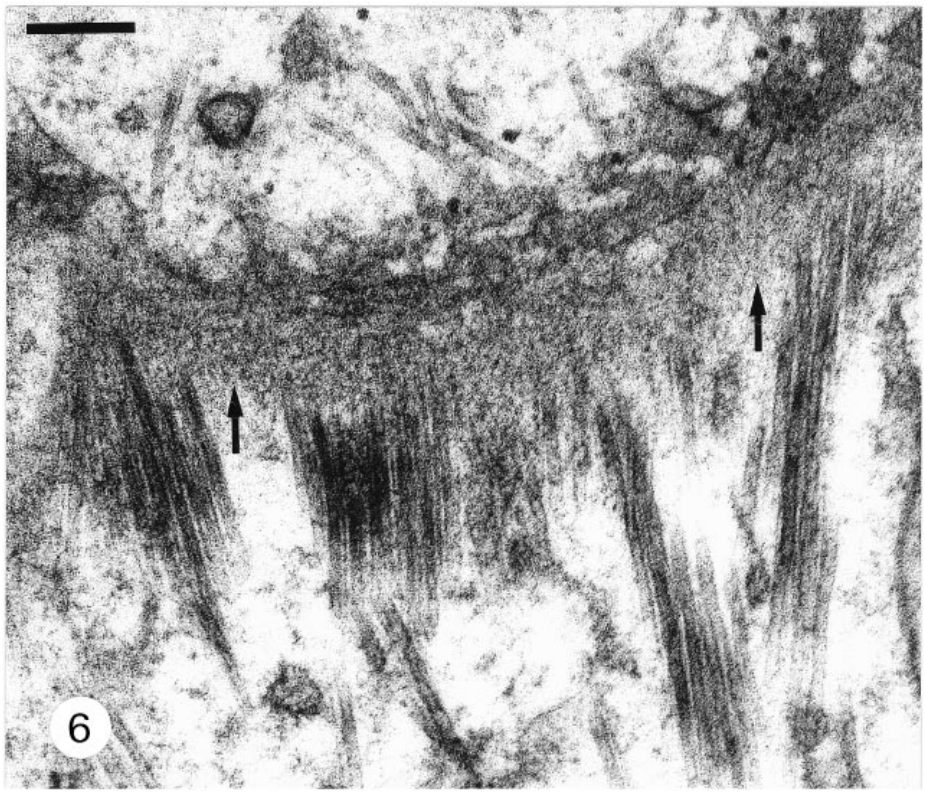

Fig. 6. A noncentrosomal cell surface-associated microtubule-organising centre. The section cuts longitudinally through the apical portions of microtubules in an array which is remotely located with respect to the centrosome in a mature guinea pig Deiters cell. The apical ends of the microtubules are associated with the cell surface in the region where it forms a concavity (see Fig. 1) that accommodates the base of an outer hair cell (towards the top of the micrograph). A meshwork of fibrous material (arrows) is concentrated around the ends of the microtubules. Bar, 0.2 μm.

ling in the centrosomal region of Deiters cells by d 3 and assembly of their remote arrays had begun by d 6. Hence, in the event that the remote arrays of supporting cells were not nucleated in their centrosomal regions but by noncentrosomal nucleating sites in the lower portions of the cells, then such nucleation was proceeding during d 6–9.

The distribution of γ-tubulin and pericentrin during the 3–9 d period was assessed by examining the binding of antibodies to these 2 proteins to frozen sections of the developing mouse organ of Corti. Sections were cut in a plane at right angles to the orientation of the hair and supporting cell rows so that they included longitudinal profiles of these cells as shown in Figure 1. MTOCs were situated at 3 levels in the organ above the basilar membrane. In the portion of the organ which has been studied, each section included 9 apically situated centrosomal MTOCs, 5 basally situated noncentrosomal MTOCs, and 5 noncentrosomal MTOCs at a more or less medial level with respect to cell apices and bases (Fig. 1). The crucial issues in this study were to determine first whether the antibodies bind to the apically situated centrosomes to establish whether the immunocytochemical procedure could detect binding at MTOCs which are known to nucleate microtubule assembly. Then, in the event that such binding occurs, the second objective was to compare the frequency of such binding with that detectable for binding at the noncentrosomal MTOCs located at the medial level (a ratio of 9:5 expected if remote arrays are nucleated at their apical ends) and at the basal level (a 9:5 ratio expected if remote arrays are nucleated at cell bases).

It was not always possible to identify which type of cell each individual antibody binding site was located in when sections were examined. This was partly because of the way in which cell apices overlap and interdigitate (Figs 1, 2). The epithelial region where supporting and hair cells were located could easily be recognised after d 8 because pillar cells and the large intercellular space between them were clearly evident in phase contrast images (compare Figs 1, 7). Generation of the outwardly directed apical cell extensions (called phalangeal processes) of mature pillar cells and the opening up of intercellular spaces at certain levels in the organ (Fig. 1) occurred mainly on d 6 and 7 (Tucker et al. 1993; Henderson et al. 1995). Prior to this, the location of hair cell rows and the epithelial region of interest could be determined because hair cell nuclei were situated at higher levels than those of supporting cells (Figs 8, 9).

During the 3–9 d period when microtubule arrays were assembling and microtubule nucleation was being effected, antibodies to pericentrin and γ-tubulin routinely stained sites at apical levels in sections of the organ (Figs 7–9) as would be expected if binding to centrosomal MTOCs was occurring. Nine discrete stained apical sites were not detectable in every section of the portion of the organ of Corti depicted in Figure 1. In most sections about 7 were evident. This shortfall may be because of failure to discriminate between centrosomes that are in close proximity to each other in neighbouring cells (Fig. 1). Furthermore, section thickness (6–10 μm) was about the same as that of cell apices (6–9 μm) so that in some cases a cell's centrosome may not have been included in a section. In contrast, no antibody binding was detected at the medial or basal levels where noncentrosomal MTOCs were located (Figs 7–9).

Evaluation of whether antibodies bind to sites at the bases of supporting cells was complicated by the presence of a layer of tympanic cells. These cells were situated immediately below the basilar membrane. The centrosome was centrally located and had a juxtanuclear position in each tympanic cell (authors' unpublished observations). Some of the tympanic cells' centrosomes were located near the lower side of the basilar membrane and were situated within 1–3 μm of supporting cell bases (Fig. 8). Hence, it was important to determine whether the binding of antibodies to sites near cell bases represents such centrosomes or basally located noncentrosomal MTOCs in supporting cells. Unequivocal discrimination between these 2 possibilities was achieved by exact spatial superimposition of each fluorescent image obtained by confocal laser scanning of a section with a phase contrast image of the same region. Such superimpositions reveal the position and level of the basilar membrane and whether sites which had bound antibody were located above (in supporting cells) or below it (in tympanic cells) (Figs 7–9). Optical sectioning through whole mounts of the organ and along the lengths of supporting cells did not permit such discrimination because the thickness and optical density of the basilar membrane throughout the developmental period in question was not always sufficiently substantial to reveal the level at which it was located in optical sections as they passed through the plane of the membrane.

Pericentrin and γ-tubulin are concentrated near the centrosomal centrioles in mature cells

There is no evidence that supporting cells are replaced during the life of a mammal. Hence, their microtubule arrays are apparently maintained for a long period

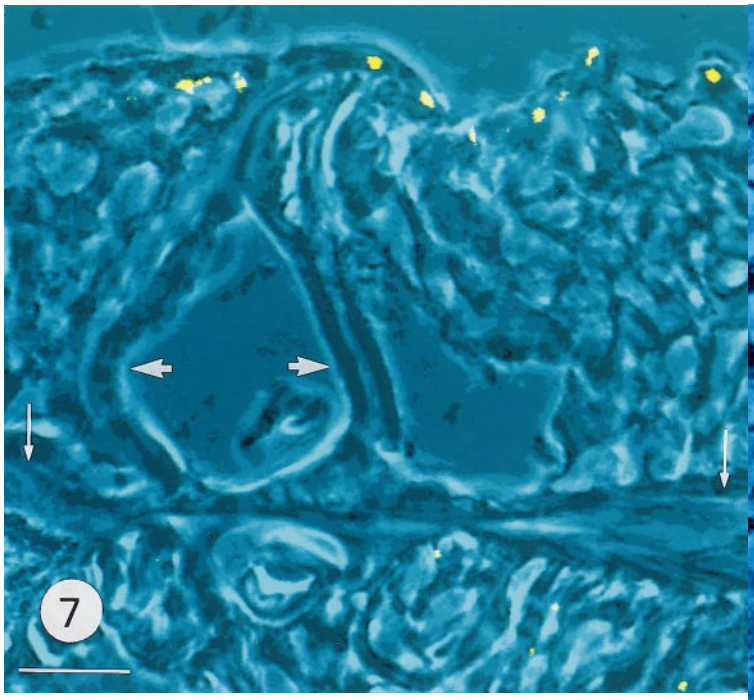

Fig. 7. Cryostat section through a 9-d-old mouse organ of Corti (oriented at right angles to cell rows as depicted in Fig. 1) after incubation with antipericentrin and a complementary fluorescein-conjugated secondary antibody. Pillar cells (large arrows) are separated by a large intercellular space (the tunnel of Corti) along most of their lengths. Pseudocolour has been used to discriminate between the fluorescent image (yellow) obtained by confocal laser scanning and the phase-contrast image (blue, the regions of greatest optical density are darkest). The basilar membrane is situated at the level indicated by the small arrows; all sites which bind antipericentrin above this level are situated near the apical surface of the organ. The other more or less circular yellow profiles are regions where antipericentrin has bound to sites in the tympanic cells below the basilar membrane. Confocal laser scanning microscopy. Bar, 10 μm.

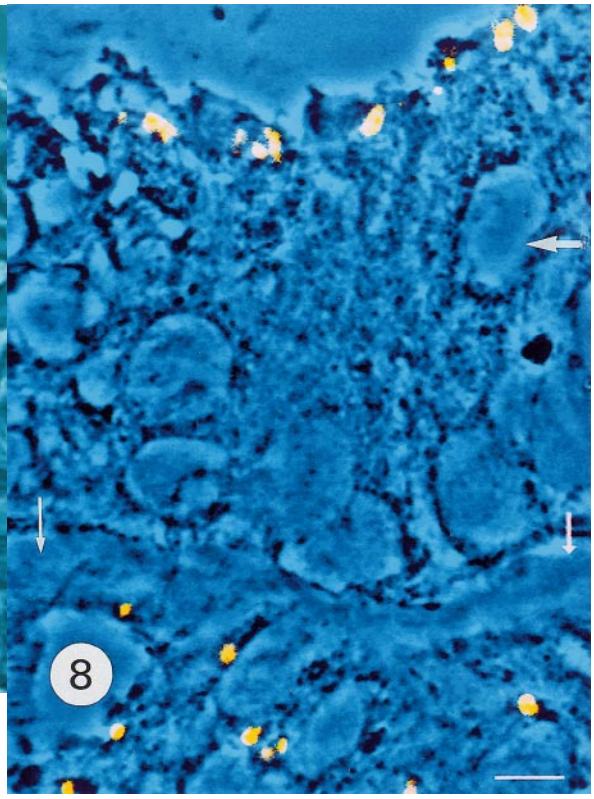

Fig. 8. Cryostat section through a 3-d-old mouse organ of Corti after incubation with antipericentrin. Specimen preparation, and image orientation and formation are as described for Fig. 7. The basilar membrane is situated at the level indicated by the thin arrows. Pillar cells are situated to the left of the nucleus (thick arrow) of an outer hair cell. Confocal laser scanning microscopy. Bar, 5 μm.

(70 y or more in the case of humans). Long-term maintenance might include nucleation of some new microtubules to replace old microtubules which have deteriorated.

Some cells were invariably distorted, displaced or torn in cryostat sections of mature organs. This was probably due to the greater degree of ossification of the bony capsule surrounding the mature cochlea which impaired the structural integrity of the sections. Antibody binding to the apices of mature cells was investigated using whole mounts of mature organs which could be more readily dissected out from the cochleas of guinea pigs than the smaller ones of mice.

Antibodies to pericentrin and γ-tubulin both bound to sites which were located in positions that corresponded to those occupied by the centrosomal centrioles. When antisera that include antipericentrin were used prior to affinity purification and at a substantially higher concentration (a dilution of 1:200) than that which selectively stained centrosomes, some staining of cell boundaries occurred which permitted unequivocal identification of the different cell types (Fig. 2); there was also staining of sites with a distribution that exactly matched that for centrosomes established using transmission electron microscopy to examine serial section sequences through cell apices in mature mouse and guinea pig organs (Henderson et al. 1994, 1995; Tucker et al. 1995; and authors' unpublished data). Centrosomes were located towards the outer side of the cell apex in each hair cell and pillar cell. Interestingly, the apices of Deiters cells exhibited an anisotropy that was 'opposite' to this; their centrosomes were towards their inner sides in most instances (Fig. 2). At some cell apices, two distinct fluorescent foci were apparent (Fig. 2); they presumably corresponded closely to the locations of a centrosome's 2 centrioles in cases where they were more widely separated than usual.

Double labelling using an anti-α-tubulin and antipericentrin or anti-γ-tubulin revealed that high concentrations of pericentrin and γ-tubulin were confined to the immediate vicinities of pillar cell centrioles. Antibody binding could not be detected near the

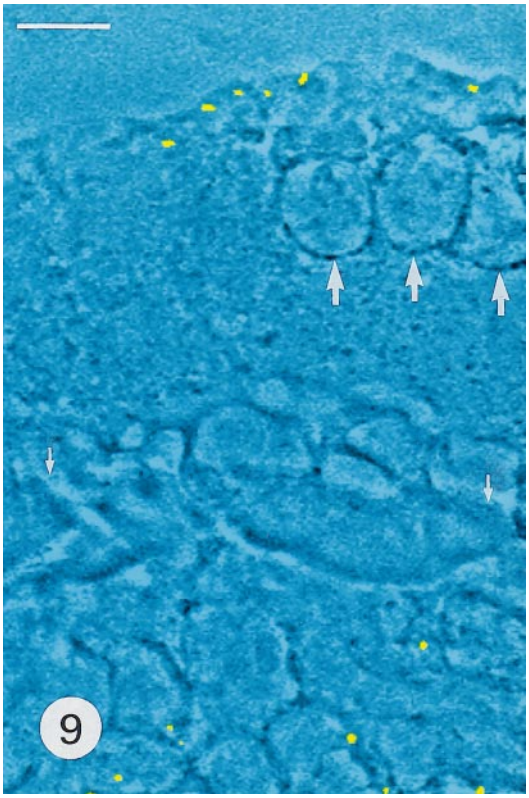

Fig. 9 . As Fig. 8 for the organ of a 7-d-old mouse after incubation with anti-γ-tubulin. Nuclei (thick arrows) in the 3 rows of outer hair cells (see Fig. 1) are included in the micrograph. Bar, 10 μm.

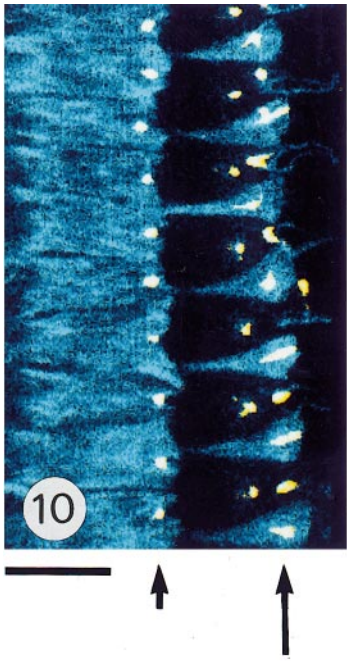

Fig. 10. Optical section through part of the apical portion of a mature guinea pig organ of Corti in the region that includes pillar cells which has been double labelled with antibodies to pericentrin and α-tubulin (using complementary secondary antibodies conjugated to fluorescein and Texas Red, respectively). Cell apices are oriented (as in Fig. 2) with their outer sides towards the right of the micrograph. Pseudocolour has been used to discriminate between sites which bind antipericentrin (white/yellow) and regions where antitubulin binding has occurred (blue). Antipericentrin usually binds to 1 discrete site near the outer side of each inner pillar cell apex (short arrow) and outer pillar cell apex (long arrow). These sites are located at the apical tips of the centrosomally associated microtubule arrays where centrosomal centrioles are situated (compare with Fig. 2). The antipericentrin binding sites situated between outer pillar cells correspond to the locations of centrosomes in adjacent outer hair cells and Deiters cells (compare with Fig. 2) which do not include large microtubule arrays oriented in the plane of the organ's apical surface (see Fig. 1). Confocal laser scanning microscopy. Bar, 10 μm.

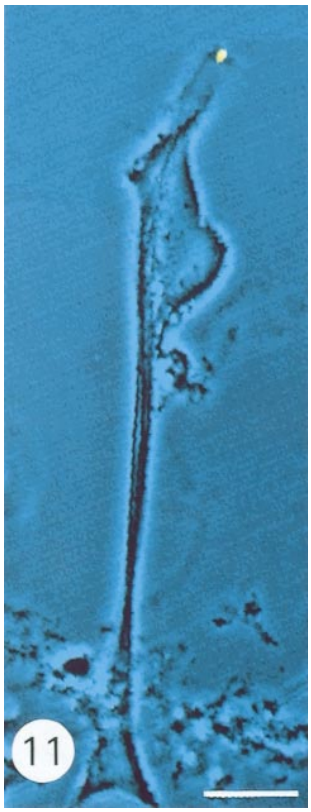

Fig. 11. Lateral view of an inner pillar cell which has been separated from a mature guinea pig organ of Corti and incubated with antipericentrin and a fluorescein-conjugated secondary antibody. Pseudocolour has been used as described for Fig. 7. The fluorescent image (yellow) has been superimposed on the phase-contrast image (blue/black). Antipericentrin only binds to a single site near the tip of the slender process at the cell apex where the centrosome is located. Confocal laser scanning microscopy. Bar, 10 μm.

apical minus ends of the large centrosomally associated microtubule arrays except in regions close to centrioles (Fig. 10).

When individual pillar cells were separated (Holley & Ashmore, 1990) from portions of organs which had been dissected out from guinea pig cochleas they could be viewed laterally. In a few instances the cells were completely intact and still attached to a fragment of the basilar membrane at their bases. Antipericentrin bound to a site near the tip of the lateral apical extension (phalangeal process) of each such cell where the centrosome was located (compare Figs 1, 11). No

binding was detected at lower levels in a cell where the ends of the remote microtubule array were situated.

None of the antibody-binding patterns described in the sections above was apparent following the omission of primary antisera. Binding did not occur either when the immunolabelling procedures were conducted with preimmune sera (isolated from the rabbits used to produce the antisera to pericentrin and *Xenopus* γ-tubulin) substituted as the source of primary antibodies. No antibody binding was detected when the affinity purified antibodies to γ-tubulin from serum R75 had been preincubated with the immunising peptide.

DISCUSSION

Minus end escape

Pericentrin and γ-tubulin are involved during microtubule nucleation in a range of cell types (see Introduction). The present study is unexceptional in so far as it shows that the sites which bind antibodies to both proteins are located at supporting cell apices where centrosomal nucleating sites are situated while microtubule nucleation is proceeding. However, these cells construct their largest microtubule arrays at locations that are remote with respect to their centrosomes. No evidence for the binding of antibodies to pericentrin and γ-tubulin at the ends of these remote arrays has been obtained while the arrays are forming. Such binding has been demonstrated at nucleating sites which lack centrioles in a range of other cell types (e.g. Gueth-Hallonet et al. 1993; Liu et al. 1993; Doxsey et al. 1994; Horio & Oakley, 1994; Debec et al. 1995). Furthermore, the procedures used for such demonstrations were similar to those employed in this study and most of the sites in question nucleate substantially fewer microtubules than those in the remote arrays of pillar cells. Hence, the case that each cell possesses only 1 (centrosomal) nucleating site, and that the microtubules which are remotely located with respect to it have escaped from it, is a reasonably strong one.

If γ-tubulin and pericentrin are present at the ends of the remote arrays then their concentrations are too low to be detected with the methods used to monitor antibody binding in this investigation. However, this interpretation implies that much of the higher centrosomal concentration represents an inactive stockpile of these proteins. Why locate such a stockpile so far from the site where most of these proteins are to be involved during microtubule nucleation? A more radical possibility is that the supporting cells do not employ γ-tubulin and pericentrin to nucleate most of their microtubules. The fact that most of the microtubules have diameters of ~ 27 nm and are composed of 15 protofilaments (rather than the conventional 13) (see Tucker et al. 1992) is relevant in this context. The case is weakened by the finding that the cells do nevertheless have centrosomal concentrations of γ-tubulin and pericentrin. Why store these proteins if they are redundant? Pillar cells have higher centrosomal concentrations of both proteins than the adjacent hair cells which nucleate much smaller numbers of microtubules (Mogensen et al. 1997). Furthermore, hair cell microtubules have diameters of 24 nm so they are presumably composed of 13 protofilaments (Tucker et al. 1992) and require γ-tubulin and pericentrin for nucleation. Importantly, the case for nucleation of the remote microtubule arrays in the apical centrosomal region receives strong support from a previous ultrastructural study of microtubule assembly in inner pillar cells. About 3000 microtubules start to assemble in the apical centrosomal region but subsequently the number of microtubules at the cell apex is reduced to about 1000 during the period that the remote array of about 2000 microtubules is being constructed (Henderson et al. 1994).

If microtubules for remote arrays are centrosomally nucleated, then a control mechanism which can count microtubules and determine how many will be released from a centrosome is also apparently needed. The ratio of microtubules in the remote and centrosomal arrays is specified with some degree of precision in both types of pillar cells (Henderson et al. 1994, 1995; Tucker et al. 1995).

If minus ends escape from centrosomes what happens to them afterwards?

Minus end capture

The remotely located arrays of supporting cells are of especial interest because both ends of such arrays are associated with sites at the cell surface. Such sites are MTOCs in so far as they are involved in the control of microtubule organisation by virtue of their association with microtubule ends (Brinkley, 1985). If the microtubules have been translocated from centrosomes, MTOCs at both ends of a remote array are capturing sites and both plus and minus ends of microtubules in a remote array are captured. It is the upper of the 2 sites in question which is the minus end-capturing site if these ends remain oriented towards the cell apex after microtubule escape from the apical centrosome.

The concept of plus end capture and evidence for its operation is well established (see Mogensen et al.

1989). It is only very recently in the context of microtubule positioning in inner pillar cells and certain other epithelial cells, that the notion of minus end-capturing sites has been advanced (Henderson et al. 1994, 1995; Meads & Schroer, 1995; Mogensen et al. 1997). Hence, nothing is yet known about the extent to which dynamic instability might be involved as minus ends seek and encounter their targets. Microtubule motors might contribute by propelling the errant microtubules downwards alongside those which do not escape, or a treadmilling mechanism (Rodionov & Borisy, 1997) might operate to effect microtubule translocation.

Analyses of events during construction of the remote arrays of pillar cells (Henderson et al. 1995; Tucker et al. 1995) indicate that escape and capture proceed as follows. Initially the errant microtubules migrate downwards. Their translocation is arrested when their basal ends make contact with the capturing site at the cell base because they can descend no further. These microtubules continue to elongate until their upper ends encounter the other capturing site which is situated at a higher level.

Meshworks of fibrous material which include β and γ-nonmuscle actin isoforms (Slepecky & Savage, 1994) are present at the capturing sites. Fibres start to accumulate as microtubule ends approach capturing sites and contact the sides of the terminal portions of the invading microtubules (Henderson et al. 1995). These meshworks may help to detain microtubule ends long enough in the vicinity of a capturing site so that capture can be consolidated by formation of robust connections to the cell surface. The fibrous material is loosely packed while microtubule ends accumulate at the surface sites and hence is unlikely to have impaired antibody access and masked γ-tubulin or pericentrin if they were present at these locations.

Control of microtubule positioning is likely to be even more elaborate than indicated in this report. A recent analysis of events at pillar cell apices has provided evidence that all microtubules at cell apices are nucleated in the immediate vicinities of the centrosomal centrioles, that this is rapidly followed by minus end release and then these recently nucleated microtubules migrate for relatively short distances to apical surface regions where docking sites (which lack γ-tubulin and pericentrin) capture and anchor their minus ends (Mogensen et al. 1997). Hence, microtubules destined for remote arrays might hop and jump to their final locations. They apparently hop from an apical pericentriolar nucleating site to an apical docking/capturing site prior to a long jump to the capturing sites at much lower levels in a cell.

The single control centre strategy for microtubule nucleation

This study has provided evidence for a strategy in which a cell controls nucleation of all its microtubules at 1 nucleating centre (the centrosome) even though most of them are destined to be located elsewhere. There is evidence that neurons and certain other cell types operate in a similar fashion (see Introduction). So far as supporting cells are concerned, it is not clear why the challenge of transporting microtubules to distant capturing sites might be preferred to that of establishing nucleating sites at particular cell surface locations. Specification of the precise locations of capturing sites is presumably just as formidable a challenge in terms of exacting reliable spatial control as it is for nucleating sites. Perhaps adherence to a 1 nucleating site : 1 cell rule is crucial during interphase if only 2 spindle poles are to result during mitosis, and is so deeply entrenched in the metazoan genome that a potentially disastrous waiver permitting more than 1 nucleating site/interphase cell has rarely evolved even in terminally differentiated cells.

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