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Tubulohelical Membrane Arrays, Annulate Lamellae and Nuclear Pores: Tripartite Membrane Architecture with the Participation of Nucleoporins

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1. Introduction

The interest in nucleoporins originates from their identification as constituents of nuclear pores. The latter are visible as prominent annuli in the electron microscope (Callan et al., 1949). Comprehensive studies carried out in the 1950s led to these general conclusions: i) all nuclear envelope (NE) have pores, and ii) irrespective of species or cell type, the pore complexes show similarities in shape and size. This very much supported the hypotheses suggesting that these pores might act as transport channels between the nucleus and the cytoplasm of eukaryotic cells. A breakthrough for these ideas was achieved by the combination of microinjection techniques with transmission electron microscopy (TEM). In 1962, Feldherr injected contrasting permeants into the cytoplasm of amebas and observed their path through pores using TEM. Together with the observation of giant polycystronic messengers in transit through nuclear pores (Stevens and Swift, 1966), these experiments determined the direction of further study in the context of nucleocytoplasmic transport.

The purpose-made transport machinery of nuclear pore complexes (NPCs), located at an interface as distinct as the NE, might be expected to be structurally unique. Surprisingly, however, the search for NPCs soon revealed quite similar annuli incorporated into reticulate cytoplasmic and nucleoplasmic membranes. For these alternative annuli-containing membranes Swift (1956) coined the term annulate lamellae (AL). In contrast to NPCs, there was no independent, strong incentive to elucidate the function of AL pore complexes (ALPCs); to date, all popular hypotheses on AL function are based on reconciling their role with the existence of nuclear pores. A critical review of the facts, however, makes clear that experimental proof for such suggestions is missing. Despite this problem and in the absence of a really comprehensive structural and biochemical comparison, equivalence of NPCs and ALPCs was nevertheless suggested (Kessel, 1981; Miller & Forbes, 2000).

Previously, the lack of knowledge on AL could be regarded as encouraging the search for their possible functions (Merisko, 1989). Richard Kessel, one of the authorities in AL

research, praised AL as the "last frontier in organelle research" (Kessel, 1992). These expectations were not met, however, and the research on AL is currently in decline. Ironically, Kessel´s publication in 1992 became the last comprehensive review on AL in an English journal, followed by the last review at all in the Russian Journal "Tsitologiia" by Morozova et al. (2005). Currently, the scarcity of publications on AL seems to have reached a point that leaves knowledge of their existence limited to a small community of experts.

Our review focuses on a third nucleoporin-containing membrane configuration, which, in conjunction with AL, has been overlooked in recent decades. Study of this enigmatic structure referred to as tubulohelical membrane array (TUHMA) (Reipert et al., 2009, 2010) might open new avenues in understanding AL in a functional context. In the following, NPCs and ALPCs are briefly summarized as annular nucleoporin configurations (Section 2). The TUHMAs are extensively introduced as novel, tubular nucleoporin configurations that differ significantly from the latter (Section 3). The formal separation into annular and tubular structures was chosen to differentiate TUHMAs as a novel player. Understanding them, however, will depend on studies of their dynamic interaction with AL, the NE and other organelles.

2. Annular assemblies of nucleoporins

NPCs and ALPCs manifest themselves in the TEM as ring-shaped pores incorporated into lipid bilayers, previously named as annuli (Feldherr et al., 1962). Their common overall morphology does not necessarily mean that they are equivalent. Studies of the fine structure by advanced EM methods, such as cryo EM and 3D-tomography, are still limited to NPCs. As a consequence, a comprehensive comparison of both structures at a fine structural level has not taken place. Also, the biochemical comparison of the nucleoporin composition of both structures has not been completed.

Here we give a short overview of both annular nucleoporin assemblies. At the same time we would like to refer to excellent overall reviews on NPCs by D'Angelo and Hetzer (2008), Lim et al. (2008), and Hoelz et al. (2011). In more detail, Wente and Rout (2010) addressed the current understanding of the transport mechanisms of the NPCs. The review by Liang and Hetzer (2011) highlights the relationship of NPCs and nucleoporins to gene regulation. Insights into the biogenesis of nuclear pores are provided by Antonin et al. (2008), and Fernandez-Martinez and Rout (2009).

2.1 The nuclear pore complex: A structure known for its major functions

NPCs are giant macromolecular ensembles which form transport channels through the nuclear membrane. They are incorporated into an annular, continuous, and strongly curved connection between the inner and outer nuclear membrane. The NPCs provide a permeable barrier between the nucleoplasm and the cytoplasm, across which only very small molecules (<30-40 KDa) can pass freely. For large soluble proteins, RNA and ribonucleo-particles the NPCs facilitate selective bi-directional transport. The macromolecules assigned for passage bear nuclear localization signals (NLSs) or nuclear export sequences. They are ushered through the nuclear pore channel with the help of transport factors, most of them summarized as karyopherins serving import (importins) and export (exportins). The driving

force for the transport is the hydrolysis of GTP by the GTPase Ran, which is maintained via a nucleocytoplasmic gradient between the two conformations, Ran-GTP and Ran-GDP (for details see: Wente & Rout, 2010). Since it turned out that nucleoporins also play critical roles in chromatin organization and gene regulation, NPCs are also under scrutiny for novel, related functions besides nucleocytoplasmic transport (Liang & Hetzer, 2011).

Despite morphological similarity and common functional aspects, there are differences in the NPCs of various species. Structural comparisons indicate that the vertebrate NPC is larger than its yeast counterpart and they also differ in molecular weight. The frequently quoted 125 MDa of a vertebrate NPC refers to estimates based on STEM analysis of NPCs of the frog *Xenopus laevis* (Reichelt et al., 1990). The weight of yeast NPCs was determined as roughly half of this weight. More recent calculations showed less dramatic differences of 60 MDa for vertebrate NPCs and 44 MDa for yeast NPCs (for review: Cronshaw et al., 2002).

Whether differences in molecular weight and size reflect modifications of the multifunctionality of pore complexes is not known. Variations in the NPC/NE organization as part of different concepts of eukaryotic cell division indicate such a possibility. Metazoa, for instance, undergo open mitosis characterized by the breakdown of the NE. The filamentous network of the nuclear lamina, otherwise tightly linked to NPCs, disassembles during this process. Yeast cells, in contrast, duplicate without NE breakdown. Interestingly, this major characteristic coincides with differences in the organization of the interface between the NPCs and the nucleoplasm of these cells. Yeast cells do not possess nuclear lamina at all (Adam, 2001). Moreover, different requirements for pore anchorage to the NE are indicated by identification of transmembrane nucleoporins in vertebrate and yeast pore complexes, which show no homology (Cronshaw et al., 2002). Here, we briefly introduce vertebrate NPCs, since they are closest in the context of finding TUHMAs in a mammalian cell line.

2.1.1 Structure

Cross-sectioned cell nuclei regularly display pore profiles in the TEM, which indicate the diameter of pore channels to be about 80 nm. If visualized *en face*, these pores appear enlarged in diameter by ring-like structures on their cytoplasmic and nucleoplasmic sides. Both the ring structures and aspects of the inner pore show a characteristic eight-fold symmetry that becomes prominent by TEM mapping based on Fourier analysis (Unwin & Milligan, 1982), and by analysis of frozen-hydrated pore complexes in the cryo-electron microscope. The "zooming in" on nuclear pores of amphibian oocytes by Unwin and Milligan highlighted details of a barrel-like pore architecture that were further elucidated in the following years by using advanced electron microscopic methods, such as high-resolution scanning electron microscopy (SEM), TEM analysis of frozen hydrated samples and electron tomography. Besides individual results of fine structural investigation, consensus has been reached over the major NPC architecture. Accordingly, the pore complex is built around a ring-like central framework that is inserted into the lipid double bilayer of the NE and anchored by trans-membrane proteins. The central framework itself is sandwiched between cytoplasmic and nucleoplasmic ring structures. Anchored to the cytoplasmic ring are filaments heading towards the cytoplasm, thereby giving the impression of individual motility. Filaments emanating from the nucleoplasmic ring,

however, are linked with a distal ring (Antonin et al., 2008). The resulting basket-like structures at the nucleoplasmic side were most clearly visible in association with a lamina network when studying amphibian pore complexes in the high-resolution SEM (Goldberg & Allen, 1992).

2.1.2 Biochemical composition

Historically, attempts to get information on the biochemical composition of NPCs were linked to efforts to isolate NPCs from mammalian NE. Based on NE isolation techniques Aaronson and Blobel (1975) prepared very distinct pore complex-lamina fractions (PCLFs) from rat liver NE, which were devoid of membranous components. The PCLF delivered detailed information on the composition of the nuclear lamina (Gerace & Blobel, 1980) and variations of lamins. Unfortunately, the tight association of the nuclear lamina with NPCs made proper separation of pores without protein losses impossible. A breakthrough in the biochemical characterization of the nuclear pore proteins was made by developing antibodies and subsequent screening of their labeling properties (Gerace et al., 1982; Davis & Blobel, 1986; Snow et al., 1987). While a number of nucleoporins could be identified this way, others remained inaccessible. Therefore, and in anticipation of equivalence between NPCs and ALPCs, AL generated *in vitro* were used as an alternative vertebrate nucleoporin source, which was known to be devoid of lamina (Miller & Forbes, 2000).

Currently, the number of proteins identified as constituents of the vertebrate NPCs includes about 30 nucleoporins (NUPs). For many of them immuno-EM, sometimes combined with differential extraction of membrane components and overlay assays, provided clues as to where they are located with respect to the pore architecture. If this is taken together with information on binding by overlays and immunoblotting it leads to a rather comprehensive view on how these nucleoporins are arranged with respect to each other (for review: Wente & Rout, 2010; Hoelz et al., 2011). Accordingly, the vertebrate NPCs are anchored to the NE by a transmembrane ring that connects to the core scaffold of the pore. This ring is built up of glycoprotein Gp210, pore membrane protein Pom121, and Ndc1. The connection between the nucleoplasm and the cytoplasm is achieved by a central tube which is anchored by linker proteins, Nup88 and Nup93. This central tube is lined and filled by a subset of NUPs containing phenylalanine-glycine (FG) repeat motives which are crucial for the translocation of cargo. The FG NUPs comprise centrally-located constituents (Nup98, Nup62, Nup54, Nup45, Nup48), as well as cytoplasmic (Nup358, Nup214, Nlp1) and nucleoplasmic constituents (Nup153, Tpr). The tube made from FG NUPs is confined by nucleoplasmic and cytoplasmic NUP-containing rings that form a core scaffold. The asymmetry in the composition of nucleoplasmic and cytoplasmic ring structures finds its continuity in filaments that extend towards the nucleoplasm and the cytoplasm. Filaments at the nucleoplasmic side are bound together by a distal ring composed of Nup153 (Panté et al., 1994), while cytoplasmic filaments provide the impression of being flexible.

Notably, whole complexes of NUPs are increasingly understood in a specific functional context. Interaction of FG NUPs of the central tube (Nup358, Nup214, Nup153, and Nup98) with mRNA export cargo, for instance, point to an essential role of these nucleoporins in mRNA export. It is thought that the Nup107-160 complex is involved in this process, too, since overexpression of specific fragments of individual components of the complex causes

marked defects in mRNA export. During mitosis, the Nup107-160 complex locates at kinetochores and spindle poles. The demonstration of its requirement for correct spindle assembly provides an interesting example for multifunctionality of complex nucleoporin ensembles.

2.1.3 Nuclear pore assembly

Metazoan cells undergo open mitosis, characterized by NE breakdown during prophase / metaphase and the reassembly of the NE during telophase. The NE breakdown is accompanied by disassembly of the NPCs. Consequently, NPCs have to reassemble as part of the formation of the nucleocytoplasmic interfaces of the daughter cells. Besides this mitosis-related process, NPCs also form while cells progress through interphase. As shown previously by Maul et al. (1972), this leads to a significant increase in NPC numbers in the interphase nuclei (doubling of pore number from 2000 to 4000 during S-phase in chemically synchronized HeLa cells).

Conditions of pore formation were previously mimicked by mixing precursor vesicle containing *Xenopus egg* extract with demembranated sperm chromatin (Lohka & Masui, 1983; Newport, 1987). As a result of an ATP- and GTP-driven process, vesicle fusion to a NE was observed which contained NPCs. Membrane fusion of the inner and outer membranes of the NE were seen as a possible starting point for pore formation. Currently, alternative scenarios are emerging (for review: Antonin et al., 2008; Webster et al., 2009), based on findings that transmembrane proteins of the NE get incorporated into ER instead of being sequestered in vesicles during mitosis (Daigle et al., 2001). Evidence was found that the contact between outgrowing ER tubules and chromatin initiates the assembly of the NE (Anderson & Hetzer, 2007). In consequence, processes related to the membrane curvature as the basis for membrane tubulation promoted interest in studies of pore biogenesis (Antonin et al., 2008). Moreover, chromatin was also identified as the site where prepores are formed after initial binding of several nucleoporins. How exactly sequestered tubular membranes and prepores find each other and are subsequently transformed into a flattened nuclear membrane is not yet clear.

For the assembly of NPCs into an intact NE, as happens during interphase of the cell cycle, several concepts have been suggested (for review: Fernandez-Martinez & Rout, 2009). The options are i) the *de novo* formation of pores in regions devoid of NPCs, ii) existing NPCs could duplicate, and iii) NPCs could assemble from cytoplasmic membranes or vesicular intermediates. Studies of pore assembly in cell free extracts and results of stable transfected HeLa cells expressing the transmembrane nucleoporin Pom121 support the idea of *de novo* biogenesis of NPCs (D'Ángelo et al., 2006). In the light of these data, it seems unlikely that cytoplasmic membranes in the form of AL play a role in NPC biogenesis. Therefore, one might wonder what functions other than serving the NPCs could be related to ALPCs.

2.2 The annulate lamellae pore complex: A structure on the search for a function

Up to the 1990s a tremendous amount of EM data was accumulated, either as short 'case reports' or in the form of systematic studies, which all provided evidence for the existence of AL in a wide variety of eukaryotic cell types and species (Kessel, 1989). Despite their

indicated omnipresence, AL were rarely encountered in TEM thin sections. This made systematic studies of the potentially new organelle difficult.

Later on, studies of AL profited from the development of antibodies against nucleoporins. Initial data indicating that ALPCs and NPCs share the central pore protein Nup62 (Dabauvalle et al., 1991; Cordes et al., 1995) were followed by more extensive immunohistochemical comparison of nuclear and cytoplasmic pores (Ewald et al., 1996). More recently, such comparative studies were complemented by observation of green fluorescent protein (GFP)-tagged nucleoporins expressed after transfection (Imreh & Hallberg, 2000; Daigle et al., 2001). Both immunohistochemistry and transfection techniques provided major technical improvements for systematic studies of AL in tissue culture cells. For the first time, the overall distribution of cytoplasmic (and also nucleoplasmic) nucleoporins became visible in the form of fluorescent spots. As demonstrated in Fig. 1, for PtK2 epithelial cells stimulated to generate cytoplasmic AL by vinblastine sulfate treatment, these spots could become large in size and strong in intensity.

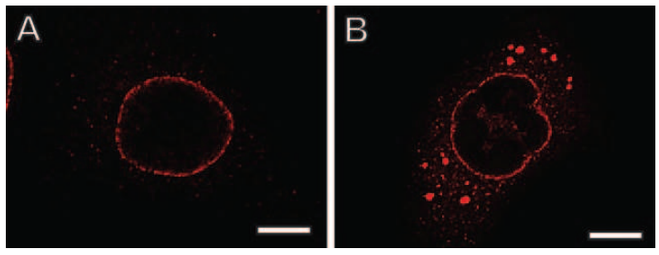

Fig. 1. Visualization of AL induced in PtK2 cells. A) Confocal section of an untreated cell displaying NPCs labeled with mAb 414. B) Treatment with vinblastine sulphate results in bright fluorescent spots in the cytoplasm reminiscent of AL. Bars, 10 μm.

Unfortunately, further technical advances were cut short by the conclusion that these spot-like labeling patterns represent AL. Whether such a correlation can be generalized or not is not yet clear, since correlative light and electron microscopy has so far spared AL as a field of research. Nevertheless, one can be confident that the fluorescently-labeled structures mostly represent AL, since it could be shown that antibodies applied at light microscopic level also labeled AL if used for immunogold labeling (Ewald et al., 1996). Accordingly, immunofluorescence microscopy was used for identification of cell lines which contain significant amounts of endogenous AL (Cordes et al., 1996). Despite this preparatory work, somatic cells were not much used in the past as models for research on AL.

Notably, isolation of AL for biochemical analysis and other studies poses a technical challenge. Generation of AL from amphibian oocytes has proven a useful strategy to overcome difficulties in isolation. In 1991, a method of generating AL cell-free from *Xenopus* egg extracts was published by Dabauvalle et al.. Further progress towards an assay was made by separation of membranous and other components that are not required for AL assembly *in vitro* by using ultracentrifugation (Meier et al., 1995). The resulting AL was separated for analysis of nucleoporins based on Western blotting. The cell-free AL formation assays were subsequently used in a couple of sophisticated experiments, which led to extension of the list of nucleoporins known to be located at both NPCs and ALPCs (Miller et al., 2000; Miller & Forbes, 2000).

A review of the publications on AL from previous years indicates that the incentive for elucidation of these structures seems to have changed. Initially, a systematic comparison of ALPCs and NPCs was seen as a way of possibly revealing any differences (Ewald et al., 1996; Cordes et al., 1996). As a result, a set of data was generated that concentrated on the similarities of both structures. To be precise, however, it should be stated that the biochemical comparison of ALPCs and NPCs is still incomplete, and that a systematic comparison of their fine structure is still at an early stage. Despite this, equivalence of NPCs and ALPCs was used as an argument in more recent publications (Miller & Forbes, 2000), and AL were referred to as NPC-containing membrane structures (Walther, et al., 2003).

2.2.1 AL structure and arrangement

AL are conspicuous in TEM thin sections, since they contain prominent annuli incorporated in reticular lipid membrane sheets (Fig. 2).

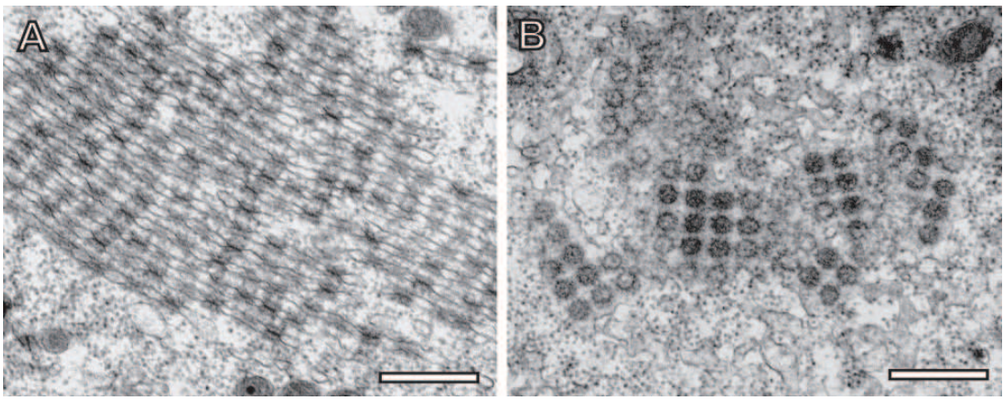

Fig. 2. AL in the cytoplasm of *Xenopus* oocytes visualized by TEM. A) Longitudinal epoxy resin section displaying an AL membrane stack endowed with numerous pore profiles. B) Transverse section displaying annuli in the *en face* view. Bars, 500 nm.

In cross sections, these annuli display a profile of about the same size as pore channels of nuclear pores. The *en face* view of the resin embedded or negatively stained annuli reveals ring structures around an inner pore channel, which are of eight-fold symmetry, quite similar to nuclear pores (Maul, 1970a; Franke et al., 1981). Taken together, conventional TEM allows the identification of AL by morphological criteria.

ALPCs are frequently densely packed thereby covering most of the membrane surface. This is particularly true for AL stacks of amphibian oocytes, as demonstrated in Fig. 3 representing a surface view on AL by scanning electron microscopy. Kessel (1989) described such an optimal use of membrane space by ALPCs as a tight hexagonal package. Notably, NPCs may sometimes reach similarly high densities by clustering of pores at the NE, as observed for nuclei of the *Xenopus* oocytes at developmental stage 2-3 (Kiseleva, unpublished results), in growing blastoderm nuclei in drosophila embryo (Gubanova & Kiseleva, 2008), and in hematopoietic cells under pathological conditions leading to cell death (Reipert et al., 1996).

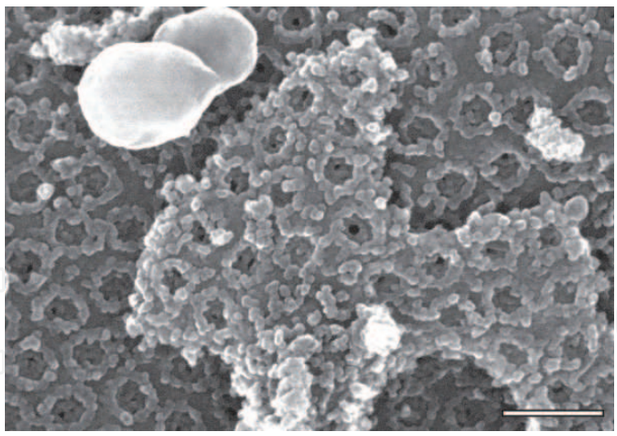

Fig. 3. Tightly distributed cytoplasmic pores of AL attached to the NE in *Xenopus* oocyte visualized by field emission in-lens scanning electron microscopy. Bar, 200 nm.

2.2.2 Occurrence and affecting factors

A review by Kessel (1989) meticulously summarized reports on the occurrences of AL in a variety of species and cell types, including observations related to pathological stages. Accordingly, AL were found in eukaryotes as different as mollusks, crustaceans, insects, amphibians, fish, reptiles, birds, plants and mammals. While many of the publications listed in this review referred to observations made in male and female germ cells, it also made very clear that the occurrence of AL is by no means restricted to them. AL was reported for somatic cells, including a variety of embryonic cells, epithelial cells, secretory cells, hormone secreting cells, muscle, and nerve cells. Kessel (1989) concluded that AL are present in all basic tissue cell types with the possible exception of some connective tissue cells.

Despite their wide-spread distribution, the likelihood of finding AL in random EM sections is rather low. This seems to be particularly true for somatic cells where the occurrence of AL might be vastly underestimated (Cordes et al., 1996). Only under conditions of metabolic activity, differentiation and rapid proliferation, does AL seem to be expressed more abundantly (Kessel 1989, 1992; Merisko, 1989). Some of these conditions are linked to diseases, including cancer, while others are part of normal cell physiological processes. Especially in germ cells and embryos, AL can be found in large amounts, organized in multiple, large stacks. Mature *Xenopus* oocytes for instance organize about 80% of all pore complexes in cytoplasmic stacks (Cordes et al., 1995). In consequence, systematic research on AL relies to a great extent on the use of oocytes and embryos.

Notably, the cellular distribution of AL can be affected by a diversity of external conditions imposed on cells and tissues. A comprehensive list of AL- affecting circumstances including drugs, culture conditions, and pathogens can be found in the review by Kessel (1992). Among the AL-affecting factors, tubulin-depolymerizing agents such as vinblastine sulfate have been proven to be effective inducers of AL in tissue culture cells (De Brabander & Borgers, 1975; Kessel & Katow, 1984; Ewald et al., 1996; Reipert et al., 2009). Given the

striking effectiveness of these agents, it was speculated that there is a possible relationship between formation of the AL network and the disassembly of microtubules (MTs) (De Brabander & Borgers, 1975; Merisko, 1989). A direct regulatory effect of tubulins on AL formation, however, has not yet been shown experimentally.

More recently, a direct metabolic link between importin β and AL formation in *Xenopus* egg extracts was reported (Walther et al., 2003) that, in the future, might be exploited for studying of AL in somatic tissue culture models. Based on a comprehensive set of experimental data, they suggested that RanGTP allows the incorporation of nucleoporins into membranes by releasing the inhibitory effect of importin β.

2.2.3 Biochemical composition

In contrast to NE, AL do not provide organelle compartmentalization. Instead, their membranes are surrounded by an exclusively cytoplasmic environment. The interface between cytoplasm and their laminar membrane sheets does not contain lamina built up from lamins, like the vertebrate nucleus (Chen & Merisko, 1988; Daigle et al., 2001). In agreement with this notion it was found that lamins are not required for assembly of AL in egg extracts. While antibodies against lamins can block formation of NPCs they do not hinder the assembly of AL stacks (Dabauvalle et al., 1991). Moreover, evidence was found that emerin, a transmembrane protein of the inner nuclear membrane, is not present in AL (Dabauvalle et al., 1999).

With the identification of nucleoporins as constituents of NPCs the question emerged whether they are also constituents of ALPCs. Labeling with the lectin wheat germ agglutinin (WGA) turned out to be positive for the O-linked N-acetylglucosamine (GlcNAc) containing moieties of glycoproteins in pore structures of both NPCs and ALPCs (Allen, 1990). More specifically, by using immunohistochemistry, immuno EM, and biochemical analysis of AL in *Xenopus* oocytes and egg extracts, the glycoprotein Nup62, a central constituent of nuclear pores, was identified as part of ALPCs (Dabauvalle et al., 1991; Cordes et al., 1995; Ewald et al., 1996; Daigle et al., 2001).

In 1996 Ewald et al. added further evidence of biochemical similarities; application of antibodies against nucleoporins located at both sides of the NPCs, namely cytoplasmic Nup180 and nucleoplasmic Nup153, demonstrated that these nucleoporins are also part of ALPCs. The nucleoporins Gp210 and Pom121, however, could not be detected as constituents of AL by this method. A molecular biological approach, based on expression of YFP-tagged Pom121 and Nup153 in tissue culture cells, however, also identified Pom121 as constituent of AL. Besides this, it confirmed previous data concerning the presence of Nup153 in AL (Imreh & Hallberg, 2000; Daigle et al., 2001). Furthermore, fluorescence recovery after photobleaching (FRAP) indicated that the turnover of Pom121 located in AL was rapid, while it was very slow in NPCs. Nup153, in contrast, seemed to be replaced continuously in both structures (Daigle et al., 2001).

Moreover, progress in biochemical analysis was achieved by studying AL formed under cell-free conditions from *Xenopus* egg extracts (Miller et al., 2000; Miller & Forbes, 2000). Aimed at identification of novel NPCs constituents by circumvention of the difficulties in

isolation of vertebrate NPCs, isolation of AL from egg extracts also led to the identification of a number of nucleoporins of *Xenopus* ALPCs. For this Miller et al. (2000) collected glycoproteins of the egg extract by using a WGA-Sepharose column, and biotin-tagged them prior to addition to the AL formation assay. As a result, Nup62, Nup98, and Nup214 were identified as constituents of the *in vitro* assembled AL. Furthermore, it was reasoned that Nup93, Nup205, and a newly identified Nup188 are present in *Xenopus* AL. Taken together this added to the evidence for similarities in the biochemical composition of ALPCs and NPCs. On the other hand, the nucleoporins identified so far represent just a fraction of those known to build up the vertebrate NPC.

2.2.4 Biogenesis

Concerning the biogenesis it is not yet clear how ALPCs are formed and from where they originate. For the latter, any suggestions are based on microscopic observations putting their cellular positioning in relation to other organelles. Accordingly, either a nuclear origin, or their formation in conjunction with rough endoplasmic reticulum (rER) were hypothesized (for review: Wischnitzer, 1970; Merisko, 1989; Kessel, 1989, 1992). Numerous reports proposed ideas in favour of one of these popular suggestions, because of their own observations of AL located proximal to the cell nucleus, or attached to rER. Also, an alternative suggestion was made based on much less frequent findings of AL linked to the Golgi complex, proposing a role of the Golgi complex in AL biogenesis (Maul, 1970b). That proposal was criticized for not being backed by EM data depicting the dynamic process related to AL formation (Kessel, 1989). In hindsight, however, all previous EM studies could only provide ideas on how biogenesis might occur. Since the observations in the EM sections occur almost randomly, there is the possibility that short but important steps in the dynamics of AL assembly were outnumbered by those observations that attracted regular attention. This seems to have happened with respect to the Golgi complex and AL. In consequence, studies of the putative relationship of both structures became sidelined.

Decades later, immunohistochemistry opened up the opportunity for a critical review of the linkage between AL and the Golgi complex. Initial double immunofluorescence labeling of AL and the Golgi complex of bovine epithelium cells, however, could not confirm any spatial relationship between both structures (Cordes et al., 1996). Based on a rather limited set of immunofluorescence data, Cordes et al. argued more generally against an intimate relationship between AL and the Golgi complexes.

An interesting aspect of AL biogenesis is its supposed coupling to the cell cycle indicated by the disassembly of AL during mitosis and their subsequent reappearance (Maul, 1970a; Erlandson & de Harven, 1971). Formation of AL as early as the telophase was confirmed later on by immunofluorescence microscopy (Cordes et al., 1996). In their morphometric studies of AL of synchronized HeLa cells Erlandson and de Harven (1971) discriminated between AL in direct continuity with rER and those showing no association with reticular membranes. From their data one could come to the conclusion that linkage with rER happens after AL is already formed, in preparation of cell division, at the S-phase of the cell cycle. Verifying the relationship of AL with respect to rER, the nucleus and other organelles during the cell cycle, therefore, could shed light on the sequence and nature of events in

which AL is involved. Such dynamics could possibly be coupled to microtubules (MTs). The latter seem to have been overlooked in systematic studies in the past despite reports of a possible association of MTs with AL (Kessel ,1992; Sutovsky et al., 1998). Perhaps, this neglect resulted from experience with MT-depolymerizing drugs, indicating that MTs are not required for AL formation itself.

3. Tubular assemblies of nucleoporins: The tubulohelical membrane array

The tubulohelical membrane array (TUHMA) was observed for the first time in the rat kangaroo kidney epithelial cell line PtK2 (Reipert et al., 2009). The initial observation was made by validating large numbers of cells for their structural preservation during rapid microwave-accelerated fixation with low concentration glutaraldehyde (Reipert et al., 2008). TUHMAs could also be observed in cryofixed and low-temperature processed samples (Reipert et al., 2009). Soon, however, it became clear that for identification of TUHMAs advanced EM preparation techniques are not required.

Initial studies indicated that TUHMAs are not very abundant within the asynchronously grown cell population of PtK2 cells. However, their single-organelle-like appearance only became obvious by immunofluorescence microscopy, using the monoclonal antibody (mAb) 414, as the most commonly used nucleoporin marker (Davis & Blobel, 1986). For successful labeling of the nucleoporins located at the tubular cores of TUHMAs, sufficient extraction of lipid material from the surrounding membranes was critical. The resulting tubular fluorescence patterns could be observed without interference from the positive cytoplasmic background, because PtK2 cells contain almost no endogenous AL (Fig. 1A), otherwise resulting in numerous fluorescent spots of various sizes. Because of both, the undisturbed view and the single-organelle-like appearance, the initial light microscopic studies could be effectively used to get hints of the extraordinary dynamics of TUHMAs.

Why did TUHMAs remain hidden to the discerning eye of electron microscopists for about half a century? We see an amalgamation of reasons: i) their transient nature, ii) their rare occurrence in thin sections, and iii) their structural hallmarks were overlooked in the two-dimensional view of TEM thin sections. In the following we provide an introduction to TUHMAs that should enable their identification in a diversity of cell types and species.

3.1 Structure

TUHMAs (Fig. 4) are lipid membrane arrays organized around tubular, proteinaceous electron-dense cores of 80 nm in diameter, confined by helix-like threads. Notably, this diameter is of about the same size as the inner pore channel of NPCs and ALPCs. The helical threads manifest themselves as zigzag-patterns resulting from their confinement within resin sections under conditions of almost parallel orientation with respect to the cutting plane. The zigzag-patterns are very clearly visible if the sections are of about the same thickness as the diameter of the core tubules of the TUHMAs. Such thin sections in the range of 80 to 100 nm are routinely produced with an ultramicrotome. It is important to note that the contrast patterns of TUHMAs cannot be explained by transverse alignment of annular pore complexes within AL membrane stacks (Hertig, 1968; Kessel, 1986).

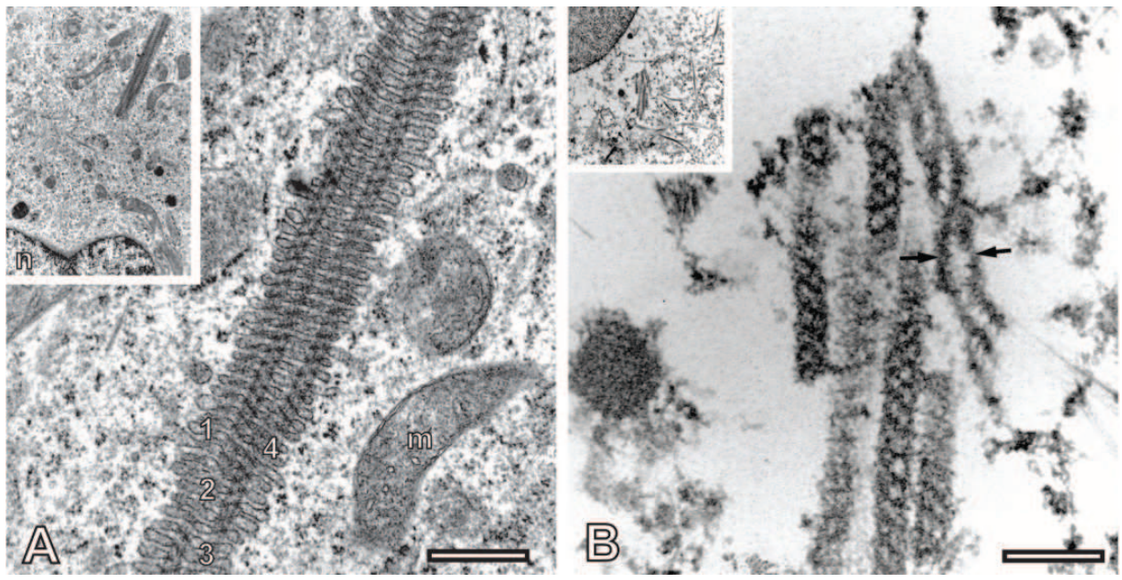

Fig. 4. TUHMA visualized by TEM. A) Aldehyde fixation followed by osmification. Tubules of uniform diameter of 80 nm, numbered 1–4, are incorporated in a regular stack of membranes and confined by helical threads resulting in black zigzag-patterns. Mitochondrion, m. B) Aldehyde fixation followed by extraction with Triton X-100 and osmification. The lipid membranes are dissolved, while the proteinaceous core tubules remain intact exposing their helix-like aspects. The core tubule on the right seems to detangle into separate threads (black arrows) (Reipert et al., 2010). Bars in A and B, 500 nm.

In agreement with our interpretation, we could follow the continuity of the helix-like threads of TUHMAs through stacks of tomographic sections (supplement in Reipert et al., 2009). Furthermore, we found that the helix-like threads lining core tubules withstand treatment of MW-fixed PtK2 cells with Triton-X100 (Reipert et al., 2010). Their helical nature was particularly apparent where disentanglement occurred (Fig. 4B). The latter indicates the possibility that the core tubule-confining threads might be double-helical in nature. However, a comprehensive analysis of the helix type is outstanding. Therefore, we use the more general terms here of helix-like or helical structures.

The core tubules provide the basis for an intermingled membrane scaffold of strongly curved lipid membranes. The overall lengths of the resulting array vary between 3-5 µm. The lipid membranes themselves are of particularly narrow curvature, and do not contain pore complexes. The nonlamellar character of these membranes was confirmed by electron tomography (see supplement in Reipert et al., 2009). Therefore, we conclude that the two major criteria characterizing AL structurally, i) ring-shaped annuli, and ii) lamellar membrane stacks (Merisko, 1989), are not applicable to TUHMAs.

3.2 Occurrence

Based on the structural definition given above, we reviewed previous publications on AL. As a result, we found a few structures resembling TUHMAs at different levels of visibility (Goldstein, 1971; Bhawan et al., 1978; Matsubara & Mair, 1979; Sun & White, 1979; Gracia-Navarro et al., 1980; Hill et al., 1984; Allen, 1988; Cheville, 1994; Alvaraz-Buylla et al., 1998).

With one exception, all authors interpreted their observations to be AL. Sun and White (1979), however, reported on a "peculiar configuration of agranular reticulum of braided channels" reminiscent of the nonlamellar membrane architecture of TUHMAs. Despite AL being the prime target of their studies, they provided an interpretation that avoided any confusion of both structures. From our point of view, their interpretation was furthered by fixation under hypotonic conditions, and very intense contrasting of lipid membranes. In consequence, the zigzag-patterns, hallmarks of TUHMAs, are less visible.

There are methods and circumstances whereby the visibility of the helix-like aspect can be enhanced. Intentional omission of membrane staining (Reipert et al., 2009), or pure contrasting of lipid membranes, both result in the almost exclusive display of helical patterns. In line with this argument, Denys Wheatley (1993, 1999) discovered helical structures in the murine fibroblast cell line 3T3, which closely resemble the proteinaceous aspects of TUHMAs. Wheatley clearly differentiated between findings described as "helical inclusions- or extrusions", and AL observed in close proximity to these structures. While this is very much in agreement with Reipert et al. (2009), it seems that the incorporation of these helical structures in complex lipid membrane arrays was overlooked by Wheatley. In consequence, he interpreted his findings as "helical pores", and postulated their extrusion from the nucleus into the cytoplasm. His attempt to substantiate this claim by immunolabeling of nucleoporins could possibly have failed because of insufficient permeabilization of the surrounding lipid membranes.

Notably, the references cited above refer to *in vivo* and *in vitro* studies in diverse somatic cells types and species. Accordingly, we would expect TUHMAs in species as different as amphibians (Gracia-Novarro et al., 1980); birds (Alvaraz-Buylla et al., 1998), and mammals (Bhawan et al., 1978; Matsubara & Mair, 1979; Sun & White, 1979; Cheville, 1994). With respect to the cell types, TUHMA-resembling structures were found in neuroblastoma cells (Goldstein, 1971), melanoma cells (Sun & White, 1979), striated muscle tissue (Matsubara & Mair, 1979), fibroblasts (Wheatley, 1993, 1999), Burkitt´s lymphoma cells (Allen, 1988), tissue of venereal sarcoma (Hill et al., 1984), hormone cells of the pituitary gland of amphibians (Gracia-Navarro et al., 1980), and neuronal precursor cells of the ventricular zone of the central nervous system of birds (Alvaraz-Buylla et al., 1998). While the majority of these examples refers to cancerous cells, the data by Gracia-Navarro et al. (1980) and Alvaraz-Buylla et al. (1997) were generated from tissues which were supposedly healthy and metabolically active by stimulated hormone secretion and neuronal differentiation, respectively.

3.3 Biochemistry

Studies of the biochemical composition of TUHMA are currently at an early stage. Isolation of TUHMAs from tissue culture has not yet been accomplished but efforts will be greatly facilitated by the availability of antibodies against nucleoporins that could serve as markers for TUHMAs in Western blotting. Notably, the mAb 414 recognizes not just one individual nucleoporin, but a whole set including the central pore protein Nup62, the nucleoplasmic Nup153, and the peripheral cytoplasmic proteins Nup214 and Nup358 (Davis & Blobel 1986; Cronshaw et al., 2002). After successful application of mAb 414 in immunohistochemistry, therefore, the question arises which of the nucleoporins stated above are indeed constituents of TUHMAs? By application of a human autoimmune serum well-characterized for

recognizing Nup62 (Wesierska-Gadek et al., 2008), we found evidence that this central constituent of porous annuli of both NPCs and ALPCs also occurs in TUHMAs. Identification of further constituents based on immunohistochemistry is currently limited by the availability of antibodies showing cross-reactivity with the marsupial species of our cell model, PtK2 cells.

Progress in studying TUHMAs can be expected by their isolation for proteomic and lipidomic analysis. Preliminary data (not shown) indicate that the cytoplasm of PtK2 cells indeed contains a nucleoporin-rich membrane fraction, clearly recognizable by Western blotting. If tested with antibodies against lamins this membrane fraction showed no indication for contamination with nuclear membranes. Therefore, and because of the almost complete absence of AL in PtK2 cells (Cordes et al., 1996; Reipert et al., 2009), the resulting signal should originate almost exclusively from TUHMAs. Following this argument, we hypothesize that all four nucleoporins recognized by mAb 414 are present in TUHMAs.

3.4 Association of TUHMAs with membrane-bound organelles

Besides information concerning the architecture of TUHMAs themselves, TEM provided insights into the association of this membrane array with other organelles. The initial data already contain surprisingly concise information on the interface between TUHMAs and adjacent organelles. Identification of nucleoporins as markers of TUHMAs greatly extended the possibilities for studying these entities in relation to other organelles, since it enables overall viewing, which incorporates a larger number of cells.

3.4.1 Association with the cell nucleus

Most remarkably, TUHMAs are able to take a polarized position with respect to the cell nucleus. Frequently, they can be found oriented either perpendicular or parallel to the nuclear membrane. Fig. 5 demonstrates these two preferential orientations of TUHMAs at light microscopic level with tubular fluorescence patterns directly attached to prophase nuclei. Correspondingly, TEM data showing TUHMAs in polarized positions with respect to the nucleus were published (Reipert et al., 2009). Besides this, polarized positioning of structures resembling TUHMAs has also been verified in previous publications (Goldstein, 1971; Matsubara & Mair, 1979; Hill et al., 1984; Wheatley, 1999).

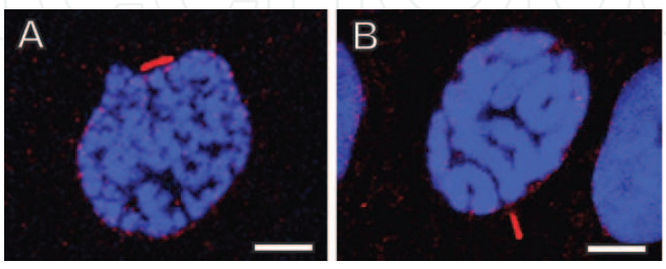

Fig. 5. Polarized positioning of TUHMAs with respect to the cell nucleus. Confocal section displaying tubular fluorescence patterns (red) labeled with mAb 414, in A) oriented parallel and in close proximity to an early prophase nucleus, and in B) perpendicular to a nucleus at a late stage of prophase. DNA counterstaining with Hoechst 33258. Bars, 5µm.

The positioning of TUHMAs close to cell nuclei in the light microscope indicates the possibility of a physical linkage. Such a linkage was confirmed by TEM serial sectioning, demonstrating that TUHMAs close enough to the nucleus are anchored via membranes to the nuclear membrane (Reipert et al., 2009). Importantly, such membrane bridges mediate the association with the nucleus regardless of the orientation of TUHMAs. Notably, these observations concerning TUHMAs differ significantly from the situation in chimpanzee oocytes. The latter displays arrays of AL with perfectly aligned ALPCs, which are linked exclusively perpendicular to the oocyte nucleus (Barton & Hertig, 1972). Both the variability in length of the nucleus-associated arrays, and the precise positioning of their ALPC columns in conjunction with NPCs, suggest the extrusion of AL from the oocyte nucleus. In contrast, TUHMAs of the somatic cell line PtK2 fused perpendicular to the nucleus show neither variation in their lengths, nor continuity of their core tubules with individual NPCs. Any TUHMAs proximal or fused to the nucleus in either direction seem to be "matured" with respect to their length, since no short fluorescent tubules could be found in this region. Therefore, we conclude that a scenario of extrusion of nucleoporins from the nucleus as part of TUHMA biogenesis does not appear to happen. Instead, we suggest that the assembly of TUHMAs occurs at some distance from the nucleus.

3.4.2 Association with ER and AL

EM revealed that TUHMAs, similar to AL, are in continuity with rER. Besides this apparent connection, TUHMAs are linked to a more extended membranous continuum. Small patches of AL were observed in association with TUHMAs; their ALPCs showed the characteristic eight-fold symmetry in the *en face* view (Reipert et al., 2009). Surprisingly, however, no immunofluorescent spots, reminiscent of AL, could be found next to TUHMAs that corresponded with the EM data. Since, otherwise, vinblastine-induced AL were clearly visible under the given preparation protocol, one may wonder what could have prevented identification of AL in its specific location next to TUHMAs. Independent of how this question may be answered in the future, the linkage of AL with TUHMAs is crucial for the understanding of both structures. In agreement with this notion, Bahwan et al. (1978), Matsubara & Mair (1979), Sun & White (1979), Gracia-Navarro et al. (1980), Hill et al. (1984), and Cheville (1994) all visualized AL in continuity with structures resembling TUHMAs. However, only Wheatley (1993) understood the necessity to differentiate between them. He made the point that there seems to be an "enigmatic relation" between helix-structures reminiscent of the core tubules of TUHMAs, and AL.

3.4.3 Association with the Golgi complex

Double labeling with mAb414 and antibodies against the Golgi matrix protein GM130 revealed that there is no fixed lateral constellation between TUHMAs and the Golgi complex within the asynchronously grown population of PtK2 cells. While most TUHMAs were located at a distance from the Golgi complex, a small fraction was linked to it. According to Reipert et al. (2010) the linkage between TUHMAs and the Golgi complex is mediated by intermediate membrane structures, named tubuloreticular structures (TRS) and AL proximal to the Golgi complex. Fig. 6 captured the rare event of getting all four structures visualized in a single TEM micrograph. If TUHMAs assemble as a result of membrane

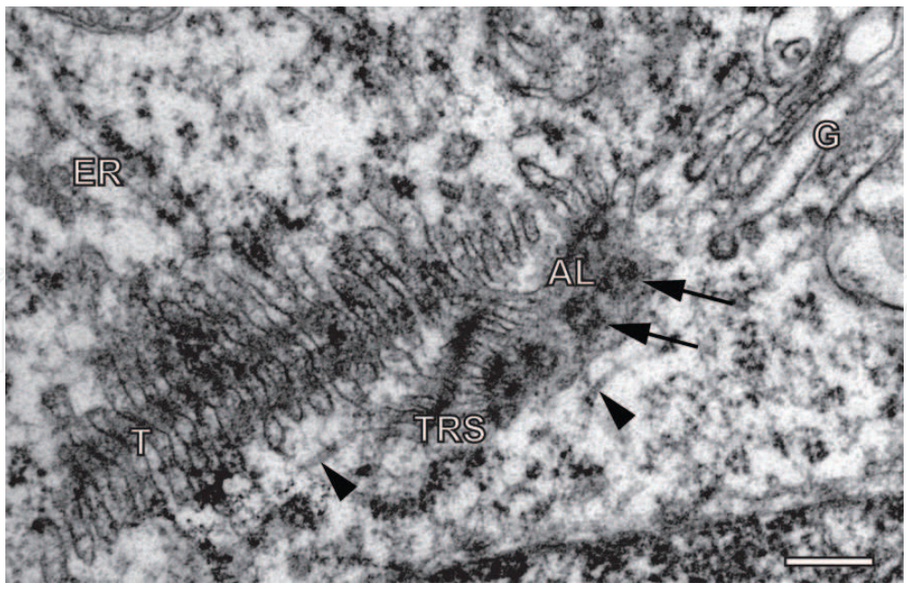

Fig. 6. The TUHMA/ TRS/ AL/ Golgi connection. A small patch of AL containing ALPCs (arrows) is located next to the Golgi complex (G). The connection between AL and a TUHMA is mediated by TRS. The TUHMA is also linked to rER. Note MTs (arrowheads) running in parallel to the axis of the TUHMA. Bars, 200 nm.

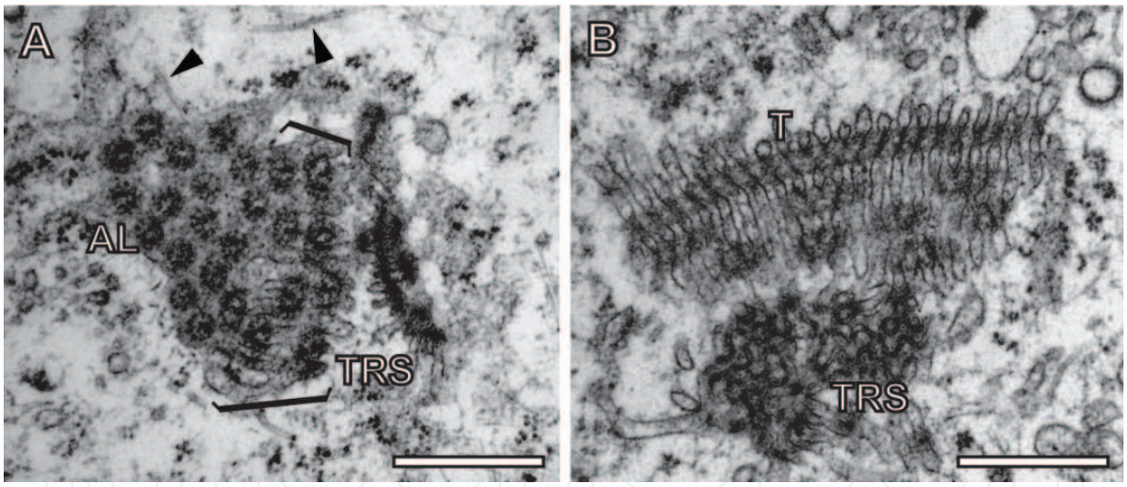

Fig. 7. TEM evidence for a possible transition of AL into TUHMAs. A) Patch of AL displaying structural intact ALPCs on its left side, and opened annuli in a transitional zone (in brackets), indicating membrane tubulation. The transition zone is continuous with TRS. Note also MTs (arrowheads). B) TUHMA linked to TRS containing darkly contrasted loops that resemble building elements of cubic membranes. Bars, 500 nm.

transformations proximal to the Golgi complex, as hypothesized previously, this should include transformation of annular nucleoporins into helical structures (Reipert et al., 2010). Fig. 7A demonstrates that AL indeed undergo a transformation. As indicated, they open up their nucleoporin-containing annuli, while at the same time the membrane sheet transforms into tubules continuously linked to TRS. The TRS themselves seem to undergo alterations

resulting in structures of a higher order. Consequently, they resemble in part a 'crystalline' lipid membrane formation, or so-called 3D-periodic, nonlamellar cubic membranes (Almsherqi et al., 2009). In conclusion, TRS, as a transitional structure linked to the Golgi complex and AL, should contain nucleoporins in an intermediate conformation before helical arrangement into TUHMAs.

3.5 Association with microtubules

On many occasions EM revealed the association of TUHMAs with MTs (Reipert et al., 2009). Therefore it seems reasonable to suggest a microtubular motor-driven dynamic being responsible for both the translocation and polarized alignment of TUHMAs with respect to the nucleus. As indicated by double labeling of TUHMAs and α-tubulin, variability in positioning of TUHMAs with respect to microtubule organizing centers (MTOCs), including those serving as basal bodies for primary cilia, is part of such a dynamic. To highlight the relationship between MTs and TUHMAs more specifically, a subset of tubulins was labeled with antibodies against detyrosinated tubulin (detyrTub), known to mark the axoneme of primary cilia and centrioles (Poole et al., 1997; Ou et al., 2003). As demonstrated in Fig. 8, it turned out that some TUHMAs showed partial co-localization with tubules built up from the longer-living form of tubulin (Infante et al., 2000). Others showed co-localization in combination with orthogonally-oriented tubules that were exclusively labeled by mAb against detyrTub. Even more striking, some axonemes of primary cilia, located in the upper section planes of the PtK2 cells, showed labeling for nucleoporin, besides labeling with Abs against detyrTub as their marker. The idea of a translocation of nucleoporins under participation of MTs was further supported by observation of single fluorescent dots of nucleoporin label in association with a small subset of primary cilia of serum starved PtK2 cells (Reipert et al., 2010). While, at first glance, the localization of nucleoporins at primary cilia and their axonemes may appear far-fetched, this could be an important aspect adding to the most recent research aiming to identify a "ciliary pore" similar to the NPC (Huang & Tsao, 2010). The current interest in such similarities was most recently inspired by evidence of the control of access of molecules to primary cilia via importin-β2 and the small GTPase Ran, and by identification of ciliary localization sequences (CLS), that, similar to nuclear localization sequences, mark the cargo for translocation (Dishinger et al., 2010).

3.6 Indications for a coupling of TUHMAs to the cell cycle

TUHMAs are apparently linked to the cycling cells, since they are almost absent in resting cell populations of so-called ´quiescent cells´ obtained by serum-starvation (Reipert et al., 2009). Accordingly, they show synchrony within individual colonies that could be followed for up to two cell divisions (results not shown). Moreover, immunofluorescence microscopy indicates that TUHMAs disappear during the course of mitosis, in a very similar way to AL (Cordes et al., 1996; Reipert et al., 2009). The latest signs for their existence can be observed in an advanced stage of prophase (Fig. 5). At this stage, TUHMAs are expected to be physically linked to the NE by membrane fusion. Their subsequent fade, therefore, very likely coincides with the disassembly of the NE and its pore complexes. In consequence, both AL and TUHMAs need to be re-assembled in the newly divided cells. While AL are already formed at late mitosis, TUHMAs would be expected to be seen much later. This can be concluded from the low number of cells containing TUHMAs that were observed in the

asynchronously grown cell population. Their presence in just ca. 5-10% of the cells would not fit with an early occurrence at the G1 phase of the cell cycle. This notion is very much in agreement with the hypothesis that TUHMA formation requires AL. If seen from the perspective of the ciliary cycle (Plotnikova et al., 2009), therefore, TUHMAs are not expected to play a role in the formation and outgrowth of primary cilia. However, they might be linked to processes of centriole duplication and ciliary resorbtion.

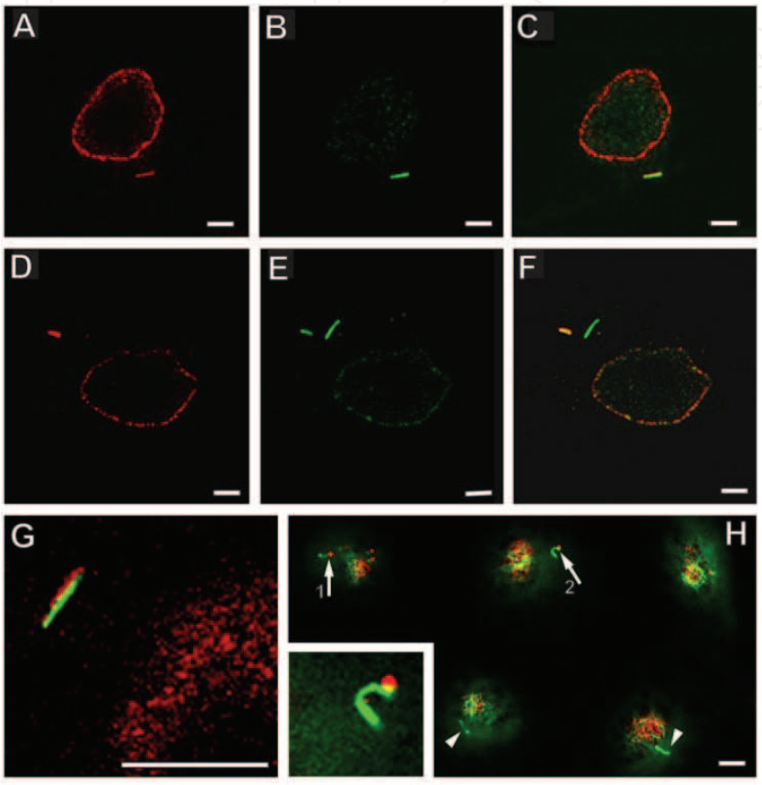

Fig. 8. Putative relation between TUHMAs and primary cilia. A) A TUHMA labeled with mAb 414 (red) and B) tubule-forming detyrTub (green) located in a confocal section close to the substratum of cell growth. C) overlay of A and B. D) TUHMA labeled with mAb414 (red) and E) detyrTub (green) forming two tubules positioned orthogonally to each other located in a confocal section close to the substratum of cell growth. F) Overlay of D and E indicating that just one of the detyrTub containing tubules co-localizes with the TUHMA in D. G) Co-localization of tubular fluorescence patterns labeled with mAb 414 and detyrTub in an upper confocal section perhaps harbouring the basal body. F) Two short cilia (arrows), numbered 1and 2, labeled with Ab against α-tubulin (green) are associated with red dots resulting from labeling with mAb 414. The insert displays cilium 2 in more detail. In contrast to cilia 1 and 2, two longer cilia in neighbouring cells (arrowheads) are free of labeling with mAb 414. (Reipert et al., 2010). Bars, 5 μm.

4. Conclusion

Taken together the initial data on TUHMAs indicate that nucleoporins are more versatile building elements than previously thought. Under as yet unknown conditions they are able

to organize in a non-annular, much more complex cytoplasmic arrangement. In the light of this finding we carried out a critical review of those structures already known to contain nucleoporins, namely NPCs and ALPCs. In the process it became apparent that the research on ALPCs lags far behind studies of the NPCs. Although this shortcoming makes a comprehensive comparison impossible, the current opinion tends to favor the equivalence of both structures. In consequence, AL are considered at best as subordinate structures serving the requirements of the NE.

Although a homeostasis involving NPCs and ALPCs was suggested, it has not so far been proven. On the contrary, recent morphometric analysis showed that the number of AL pores did not decrease to compensate for the growing number of nuclear pores during syncytial development of *Drosophila* embryos (Onischenko et al., 2004). Moreover it was found that during development of the *Drosophila* syncytium NPCs and ALPCs undergo a parallel regulation of their assembly/disassembly driven by the mitotic trigger Cdk1 and protein phosphatases (Onischenko et al., 2005). These studies are in line with previous observations of AL formation happening in parallel with the insertion of NPCs in the NE of pronuclei during fertilization of mammalian oocytes (Sotovsky et al., 1998). Taken together, these results neither indicate quantitative balancing between NPCs and ALPCs, nor accumulation of AL as a result of excessive nucleoporin synthesis. Proximity and sometimes even fusion of AL with the NE, however, point to the necessity of verifying their relationship to each other in future studies. A role of their connection in regulatory functions is indicated by observations that reported the accumulation of M-phase-promoting factor (Cdc2/Cyclin B2) in colocalization with AL in *Xenopus* oocytes (Beckhelling et al., 2003).

Alternative suggestions, which propose functions specific to ALPCs, have just recently emerged. Boulware and Marchant (2008) sought ALPC function in the context of differential regulation of cytoplasmic Ca^{2+} signaling. Their concept is based on the assumption that the numbers of ALPCs incorporated into reticulated cytoplasmic membranes affect the Ca^{2+} stores. As indicated by EM, however, function might also be related to the qualitative transition of AL into new structures in the form of TUHMAs. The supposed transformation of AL into TUHMAs manifests itself in the form of intermediates, so-called TRS. This finding might have some important implications:

1. Cytoplasmic annuli could possibly just be intermediates, which serve in the formation of structures of a higher order. Anticipating the energy needs for creation of highly ordered structures, a functional purpose appears to be reasonable. Peculiarities such as the combination of proteinaceous and lipid nanostructures, polarized orientation, single-organelle-like appearance, and a seemingly final destination of TUHMAs linked to the nuclear membrane, all indicate a yet unknown purpose.
2. The unique structure of TUHMAs is attracting the attention of biophysicists since the anisotropic, nanoperiodic design might serve in yet unknown concepts of cell communication and signaling (Reipert et al., 2010).
3. Since TUHMAs have not been visualized in germ cells so far, it appears reasonable to suggest that they exert functions exclusively in somatic cells. The indicated correspondence of TUHMAs with primary cilia found in PtK2 cells does not necessarily mean their occurrence is restricted, since primary cilia are omnipresent in a diversity of somatic cell types. Whether TUHMAs could serve particular functions in a subset of ciliated cells, as suggested for cells undergoing oriented cell division (Reipert et al.,

2010), awaits verification. Notably, observations of TUHMAs in a kidney epithelial cell line, namely PtK2, their occurrence in stratified muscle (Matsubara & Mair, 1979), as well as the finding of TUHMA aberrations in melanoma cells (Reipert et al., unpublished), all point to cells derived from tissues with well-defined cell division axes. Finding out how such division axes are maintained during morphogenesis still poses a challenge for current research. Since there is increasing evidence for the implication of defects in oriented cell division in polycystic kidney diseases and tumor genesis, this should be an incentive for investigation of possible nanophysical properties of TUHMAs that might be able to sense direction (Reipert et al., 2010).

4. Since both AL and TUHMA show a linkage to the cell cycle, studies of AL in this context have to be resumed and combined with the elucidation of TUHMA biogenesis. Particular attention has to be paid to a possible role of the Golgi complex that has been sidelined in research on AL in the past. Saraste et al. (2009) raised the interesting question whether an intermediate compartment (IC) expresses its own functional identity between the ER and the Golgi complex by carrying out or participating in some cellular functions. From our point of view, TUHMA biogenesis could be perceived as the dynamics of an IC between the Golgi complex and the rER that might result in a functional organelle.
5. Initial studies of TUHMAs indicate the importance of MTs for their existence and dynamics. TUHMAs disintegrate under the very same MT-destabilizing conditions under which AL are generated (Reipert et al., 2009). Both polarization and translocation of TUHMAs are processes that are most likely linked to MTs. Moreover, MTs could play an active role in the transformation of AL into TUHMAs by organizing the IC in conjunction with the Golgi complex. Temporal co-localization of TUHMAs with detyrosinated tubulin, as well as indications for the translocation of nucleoporins with respect to primary cilia (Reipert et al., 2010) could indicate the direction of future functional studies.
6. Initial evidence indicates that TUHMAs are formed away from the side of their final destination at the nuclear membrane. Consequently, they would have to undergo a three-step process: i) assembly next to the Golgi complex, ii) translocation towards the nucleus and oriented alignment with respect to the nuclear membrane, and iii) fusion of their membranes with the NE. Importantly, the latter does not include the fusion of core tubules with individual NPCs, which otherwise could easily be interpreted in the context of the nucleocytoplasmic transport. Instead, the fusion results in an extraordinary extension of the nuclear membrane lumen by the lumen of the membrane array. If the purpose of this extension is related to nucleocytoplasmic transport, a boost in the transport rate could possibly be achieved via a yet unknown luminal pathway that regulates NPC function.
7. Fluorescence microscopic observations interpreted as AL have to be handled with more care than previously, since intermediate structures, such as TRS, are likely to contribute to the fluorescent spots seen in the light microscope. Moreover, the immunofluorescence data have to be interpreted with caution because they depend on membrane permeabilization, and perhaps on the status of the nucleoporin organization itself. Notably, patches of AL, regularly seen next to TUHMAs in the electron microscope, were not detected by immunofluorescence microscopy, while AL induced by vinblastine sulfate treatment could be visualized using the very same protocol

(Reipert et al., 2009). Since nucleoporin markers do not seem to comprehensively mirror processes in the cytoplasm, correlative microscopy, comparing fluorescence patterns with EM fine structures is required in future studies.

The initial data on TUHMAs already indicate that these enigmatic structures do not have a life cycle independent of the other two nucleoporin-containing membrane configurations. Understanding of their possible function in the future, therefore, will require the elucidation of the tripartite nature of nucleoporin organization. Whether progress can be made in these studies will strongly depend on overcoming dogmas built up by previous research on AL, in particular concerning their relation to the Golgi complex and MTs.

Besides revealing their functions, the elucidation of TUHMAs will help to answer questions concerning the relationship between self-assembly of complex structures and gene expression. What governs the transformation of the proteinaceous pore assembles into helical structures? Is the tubulation of lamellar lipid membrane sheets driven by proteins known for their capability to curve membranes, such as reticulons and Yop1/DP1 (Voeltz & Prinz, 2007; Kiseleva et al., 2007), or do the nucleoporins themselves play a decisive role in membrane curvature? How might the cellular lipid composition affect the formation of nonlamellar membrane arrays and the nucleoporin architecture? Answers to these questions will expose "nanotechnologies" of the cell, which have previously gone undiscovered.

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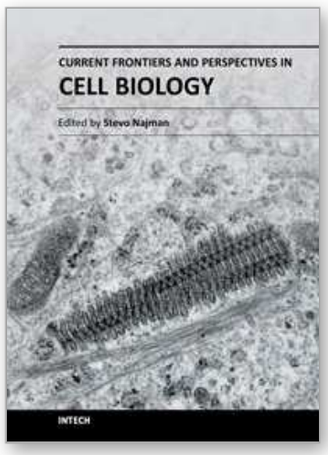

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