

Outer dense fibre protein 2 (ODF2) is a self-interacting centrosomal protein with affinity for microtubules

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Summary

Outer dense fibre protein 2 (ODF2) is a major protein of sperm tail outer dense fibres which are prominent sperm tail-specific cytoskeletal structures. Moreover, ODF2 was also identified as a widespread component of the centrosomal scaffold and was found to associate preferentially with the appendages of the mother centriole [Nakagawa, Y., Yamane, Y., Okanou, T., Tsukita, S. and Tsukita, S. (2001) *Mol. Biol. Cell* 12, 1687-1697]. Secondary structure predictions indicated ODF2 as an overall coiled-coil protein with a putative fibre forming capacity. To investigate its potential functions in generating the centrosomal scaffold and in microtubule nucleation we asked whether ODF2 is able to form a fibrillar structure by self-association *in vivo* and if it interacts with microtubules.

By cytological investigation of transfected mammalian cells expressing ODF2-GFP fusion proteins and *in vitro* coprecipitation assays we could demonstrate that ODF2 is a self-interacting protein that forms a fibrillar structure partially linked to the microtubule network. Microtubule cosedimentation and coprecipitation assays indicated ODF2 as a microtubule-associated protein. However, we could not demonstrate a direct interaction of ODF2 with tubulin, suggesting that binding of endogenous ODF2 to the axonemal as well as to centrosomal microtubules may be mediated by, as yet, unknown proteins.

Key words: ODF2, Outer dense fibre, Centrosome, Microtubule associated protein, Intermediate filament protein

Introduction

Outer dense fibre protein 2 (ODF2) is a major protein of sperm tail outer dense fibres (Brohmann et al., 1997; Shao et al., 1997; Turner et al., 1997; Schalles et al., 1998). The outer dense fibres (ODF) are prominent sperm tail-specific cytoskeletal structures. They consist of nine fibres that accompany the tubuli doublets of the axoneme on its outer edge. At the anterior end of the sperm tail the ODFs make close contact with the paracentriolar connecting piece and extend posteriorly into the principal piece (Fawcett, 1975). ODFs are found in the sperm tails of animals with internal fecundation, and are conserved across the animal phylogenetic tree, including insects (Baccetti et al., 1973). Up to now no active motility could be assigned to these fibres. Instead of being directly involved in the induction of progressive motility the ODF seem to have a modulating influence on sperm motility. They may be necessary for the maintenance of the elastic properties of the sperm tail and may provide tensile strength that is necessary to protect the sperm tail against shearing forces encountered during epididymal transport and especially during ejaculation (Baltz et al., 1990). In addition, they may also support the axonemal beat by force transmission to the flagellar base (Lindemann, 1996). The widespread occurrence of ODFs in the sperm tails of animals with internal fecundation supports their importance for sperm morphology and function (Mortimer, 1997), and is in accordance with evolutionary conservation of ODF proteins in mammals (Burfeind and Hoyer-Fender, 1991; Gastmann et al., 1993; Hoyer-Fender et al., 1995; Brohmann et al., 1997; Hoyer-Fender et al., 1998; Petersen et al., 1999).

The ODFs are composed of at least 14 polypeptides (Vera et al., 1984; Oko, 1988; Petersen et al., 1999) of which only a few have been identified. ODF2 proteins consist of about 590 amino acids with a deduced molecular mass of about 70 kDa (Brohmann et al., 1997; Hoyer-Fender et al., 1998; Petersen et al., 1999). In the C-terminal region (at amino acid positions 392-413 and 530-551 of rat ODF2) two leucine zipper motifs are present which are responsible for interaction with the leucine zippers of ODF1 (Shao et al., 1997). Secondary structure prediction (Lupas et al., 1991) indicated ODF2 as an overall coiled-coil protein. Based on the properties of the known ODF proteins and their predicted secondary structures we have suggested that first a fibrillar scaffold is formed by fibre forming proteins which is then stabilized by crosslinking of the fibrils to build the higher order fibre structure of ODFs (Petersen et al., 2002).

ODFs are very stable structures, which could be isolated by disintegration of all other sperm tail structures (Vera et al., 1984). In addition, the highly insoluble nature of ODFs together with the secondary structure prediction of ODF2 classified ODF2 as a putative intermediate filament protein. As is known from sperm tail disturbances in humans (Éscalier and David, 1984) the formation of outer dense fibres depends on axonemal microtubules but it is not known if there is any interaction between these structures.

ODF2 is not restricted to male germ cells as it has been identified as a widespread scaffold component of the centrosome, the microtubule (MT)-organizing centre of the cell (Nakagawa et al., 2001). Centrosomes determine the polarized

organization of MTs in interphase cells and play a central role in organizing the mitotic spindle to separate chromosomes in mitotic cells (for reviews, see Kellogg et al., 1994; Stearns and Winey, 1997; Zimmerman et al., 1999). In most animal cells centrosomes are composed of a pair of centrioles and a surrounding electron-dense cloud of pericentriolar material (PCM) (Bornens et al., 1987). ODF2 is associated with mother centrioles in a cell cycle-dependent pattern especially with the distal appendages (Lange and Gull, 1995; Nakagawa et al., 2001).

In order to verify the predicted fibre-forming capacity of ODF2 and to investigate whether ODF2 associates with microtubules, ODF2-GFP fusion constructs were transfected into *cos-7* cells and their subcellular localization pattern was analysed. ODF2 was shown to form a fibrillar network that associates partially with the microtubular network. Copurification and cosedimentation assays revealed that ODF2 is a self-interacting protein associated with microtubules. However, no direct interaction between ODF2 and tubulin could be found, suggesting that association of ODF2 with the microtubular cytoskeleton as well as with the centrioles may be mediated by other as yet unknown proteins.

Materials and Methods

Cell line

Cos-7 cells (ATCC) were grown in DMEM, 10% foetal bovine serum, 5% penicillin/streptomycin (all from Gibco) at 37°C in 5% CO₂.

Antibodies

The following antibodies were used: anti-ODF2 (Brohmann et al., 1997), anti-GFP (Molecular Probes, Eugene, OR, USA), anti- α -tubulin (Calbiochem, San Diego, CA, USA), anti-rabbit Alexa 488 (Molecular Probes, Eugene, OR), anti-mouse Cy3, peroxidase-conjugated anti-rabbit IgG and peroxidase-conjugated anti-mouse IgG (all Sigma Biosciences, St Louis MO, USA).

Subcloning of *Odf2* into pEGFP-N1 and transfection

Odf2 sequences were amplified by PCR and ligated in pEGFP-N1 (Clontech, Palo Alto). Cloning of all ODF2 constructs in the correct reading frame to the open reading frame of EGFP were verified by sequencing. For generation of the full length rat *Odf2* clone (ODF2NC) amplification with primer pair Odf2N (CAAGCTTC-GAATTCGAATGAAAGGGTACACCGTG)/Odf2C (TGGATCCC-GGGTCCGTAAGCGGGCCCTGC) was performed. The C-terminal region (ODF2N2C) was amplified with Odf2N2 (TC-GAATTCGTATGGAAGCTTTATCTACTCTG)/Odf2C. Two N-terminal fragments (ODF2NC1 and ODF2NC2) were generated with the primer pairs Odf2N/Odf2C1 (TGGATCCC-GGGGTCTCTCTAC-CAAGCTGTG) and Odf2N/Odf2C2 (TGGATCCC-GGGGTAGATAAGCTTCAGC), each primer containing restriction enzyme recognition sequences for cloning. The 5' region was cut with *EcoRI*, the 3' region was cut with *SmaI*, and PCR products were cloned into *EcoRI/SmaI*-cut pEGFP-N1. Constructs were electroporated into *cos-7* cells according to the method of Van der Hoff et al. (Van der Hoff et al., 1992) with modifications.

Self-association assay

Full-length ODF2 (ODF2NC) as well as the two N-terminal fragments (ODF2NC1 and ODF2NC2) and the C-terminal ODF2 fragment (ODF2N2C) were subcloned into pET-41c (Novagen) (Studier and

Moffat, 1986). Constructs were transformed into *E. coli BL21* and protein expression induced by addition of IPTG (isopropyl β -D-thiogalactoside). Bacteria were lysed by sonification and freezing.

Cos-7 cells transfected with ODF2-GFP constructs were washed in PBS, taken into lysis buffer (50 mM sodium borate, pH 8.0, 150 mM NaCl, 1% Nonidet-P40, 0.5% sodium deoxycholate, 0.1 mg/ml PMSF, 1 μ g/ml aprotinin, 1 μ g/ml leupeptin) and lysed by sonification.

The bacterial cell lysate and the *cos-7* cell lysate were mixed, incubated with glutathione-Sepharose beads, and GST fusion proteins, as well as associated proteins, were isolated according to the manufacturer's protocol (Amersham Pharmacia Biotech).

Microtubule cosedimentation assay

Purification of microtubules and microtubule-associated proteins (MAPs) were performed according to the methods of Vallee and Collins (Vallee and Collins, 1986) and (Kellogg et al., 1989). In brief, transfected *cos-7* cells were washed first in PBS and then in PEM-1 (0.1 mM Pipes, pH 7.6, 1 mM EGTA, 1 mM MgSO₄). Cells were resuspended in cold swelling solution (1 mM EGTA, pH 7.6, 1 mM MgSO₄) and homogenised after addition of protease inhibitors (final concentrations: 10 μ M benzamidine-HCl, 10 μ g/ml aprotinin, 10 μ g/ml leupeptin, 10 μ g/ml pepstatin). After addition of Pipes (final concentration 0.1 M) and centrifugation for 5 minutes at 700 g, the supernatant was aspirated and supplemented with DTT (0.5 mM), GTP (1 mM) and taxol (20 μ M) and incubated at 37°C for 2 minutes. The mixture was centrifuged at 48,000 g for 30 minutes at 4°C onto a sucrose cushion (10% sucrose in BRB80X: 80 mM Pipes pH 6.8, 1 mM MgCl₂, 1 mM EGTA, 0.5 mM DTT, 1 mM GTP, 5 μ M taxol, 10 μ M benzamidine-HCl, 10 μ g/ml aprotinin, 10 μ g/ml leupeptin, 10 μ g/ml pepstatin). The pellet was resuspended in BRB80X and again centrifuged at 45,000 g for 15 minutes at 20°C. The resulting pellet was boiled in SDS sample buffer. As a control the same experiment was performed with the following modifications: the supernatant after the first centrifugation step was supplemented with DTT (0.5 mM) only and the mixture incubated at 4°C for 30 minutes. High-speed centrifugations were then performed as described but omitting both GTP and taxol in BRB80X solutions. The final pellet was boiled in SDS sample buffer and analysed by SDS-PAGE.

Tubulin copurification assay in *Xenopus* oocyte extracts

Preparation of *Xenopus* oocyte extracts were modified after Sawin and Mitchison (Sawin and Mitchison, 1991), Murray and Kirschner (Murray and Kirschner, 1989) and Stearns and Kirschner (Stearns and Kirschner, 1994). In brief, *Xenopus* oocytes were washed in HSB (10 \times HSB: 110 mM NaCl, 2 mM KCl, 1 mM MgSO₄·7 H₂O, 0.5 mM Na₂HPO₄·12 H₂O, 2 mM NaHCO₃, 15 mM Tris-HCl, pH 7.4), dejellied in NAT (110 mM NaCl, 20 mM Tris-HCl, pH 8.5, 0.1 mM EGTA, 5 mM DTT) and again washed in 1 \times HSB. Oocytes were then washed in BRB (80 mM K-Pipes, pH 6.8, 1 mM MgCl₂, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, 2 μ g/ml aprotinin, 2 μ g/ml pepstatin, 1 μ g/ml leupeptin, 0.5 μ M cytochalasin D) and crushed at 10,000 g for 10 minutes at 4°C. The cytoplasmic fraction was purified by repeated centrifugation steps and stored frozen at -80°C.

ODF2-GST fusion proteins were expressed in *E. coli* and purified on glutathione-Sepharose beads according to the manufacturer's protocol (Amersham Pharmacia Biotech). Purified fusion proteins were incubated in 50 μ l of *Xenopus* oocyte extracts diluted with 80 mM Pipes, 1 mM MgCl₂, 1 mM EGTA, 1 mM GTP, 10% glycerol, pH 6.8 to a total volume of 200 μ l, and supplemented with 250 ng/ μ l of tubulin (purified from bovine brain; Molecular Probes, Eugene, USA). Incubation was performed at 35°C for 30 minutes in the presence of glutathione-Sepharose beads. Beads were precipitated by centrifugation and washed three times in PBS. Bound proteins were

eluted from the beads by the addition of reduced glutathione and the beads separated from unbound proteins by centrifugation. The supernatant was carefully aspirated to avoid contamination by the pellet and used for SDS-polyacrylamide gel electrophoresis and western blotting. A control experiment was performed under the same conditions but without the addition of purified ODF2-GST proteins. A very weak tubulin band was found in the fraction after elution with glutathione but not before 45 minutes exposure of the chemiluminescence to a film whereas the result presented in Fig. 6B was found even after 2 minutes exposure.

Binding assay for direct interaction of ODF2 with tubulin

Purified ODF2-GST fusion proteins were incubated with 10 μ g tubulin (purified from bovine brain; Molecular Probes, Eugene, USA) in the presence of glutathione-Sepharose beads in a total volume of ~500 μ l under stabilising or destabilising conditions. To promote microtubule formation proteins were incubated in BRB80X (containing 10% glycerol and 5 μ M taxol) at 37°C (stabilising conditions) for 45 minutes. Destabilising conditions were incubation in PEM-1 (containing 10 μ g/ml aprotinin, 10 μ g/ml leupeptin, and 10 μ g/ml pepstatin) at 4°C for 45 minutes. GST fusion proteins and associated proteins were isolated by affinity binding to glutathione-Sepharose beads, elution and separation from the beads by the addition of reduced glutathione and centrifugation. Eluted proteins were separated by SDS-PAGE, transferred to Hybond-C and probed with antibodies.

Western blotting

Proteins were separated on SDS-polyacrylamide gels (Laemmli, 1970) and transferred to Hybond-C (Amersham Pharmacia Biotech, Freiburg, Germany) (Towbin et al., 1979). The membrane was blocked in 5% dry milk in TBST (10 mM Tris-HCl pH 7.6, 150 mM NaCl, 0.05% Tween 20), and incubated with the primary antibodies in blocking solution. Bound antibodies were detected via binding of the secondary antibody (anti-rabbit IgG or anti-mouse IgG) linked to horseradish peroxidase (Sigma Biosciences, St Louis) and chemiluminescence (Renaissance Enhanced Luminol Western Blot Chemiluminescence Reagent Plus; NEN Life Science Products, Zaventem, Belgium).

Immunocytochemistry

Transfected cells were grown on coverslips, fixed either in methanol or with 3.7% paraformaldehyde in PBS (phosphate-buffered saline) at pH 7.5, incubated in 50 mM NH₄Cl, and washed in PBS. Cells were preincubated in 0.2% Triton X-100 in PBS for 5 minutes, washed in PBS, and incubated in 0.2% bovine serum albumin, 0.1% Triton X-100 in PBS for blocking unspecific binding sites. Incubation with the primary antibodies were done at 4°C overnight. DNA was counterstained with DAPI. Images were taken by confocal microscopy (LSM 510, Zeiss), and processed using Adobe Photoshop 5.0.

Results

Localisation of ODF2-GFP fusion proteins in transfected cells

The coding sequence of *Od2* was ligated in frame to the coding region of the green fluorescent protein in pEGFP-N1 resulting in fusion proteins with a C-terminal GFP tag. Four different ODF2-GFP fusions were produced in order to determine the significance of the leucine zipper motifs positioned in the C-terminal region of ODF2 (Fig. 1A). The full-length ODF2-GFP (ODF2NC) encodes a fusion protein of about 97 kDa. Two C-terminal truncated proteins were

produced: ODF2NC1 contained one of the two leucine zippers and resulted in a fusion protein of about 77 kDa, ODF2NC2 did not contain any of the leucine zipper motifs and resulted in a fusion protein of about 70 kDa. The N-terminal truncated protein ODF2N2C contained both leucine zippers and encoded a fusion protein of about 52 kDa. The fusion constructs were transfected into cos-7 cells and their subcellular localisation determined from the GFP fluorescence. There was slightly more ODF2NC in the nucleus. About 37% of cells showed a brighter nuclear than cytoplasmic fluorescence, about 30% of cells showed no nuclear localisation and in ~33% of the cells cytoplasmic and nuclear staining was the same [total number of cells investigated for quantification (*n*) 115]. Regarding the C-terminal truncated proteins, ODF2NC1 showed a preferential cytoplasmic localisation, with 78% of cells showing exclusive cytoplasmic fluorescence and 22% of cells showing no difference in fluorescence between nucleus and cytoplasm (*n*=234). ODF2NC2 is predominantly located outside the nucleus: 96% of all cells showed a bright fluorescence of the cytoplasm and a dark nucleus (total amount of cells: *n*=168). The N-terminal truncated protein ODF2N2C showed preferential nuclear localisation: 73% of cells showed a bright nuclear fluorescence whereas 26% of cells showed a similar fluorescent intensity of the cytoplasm and the nucleus (although the nucleus is always slightly more prominent; *n*=242) (Fig. 1B). The subcellular distributions of all ODF2-GFP fusion proteins are shown in Fig. 2.

Colocalisation of ODF2-GFP fusions with the cytoskeleton

ODF2NC-GFP showed partial colocalisation to the microtubule network (Fig. 2A-C). Association to microtubules is also evident from the localisation to the mitotic spindle (Fig. 2D-F, arrows). The overall appearance of ODF2NC resembles a fibrillar structure (Fig. 2A,G, arrows) although not as prominent as the microtubular fibres. A fibrous appearance is also evident in ODF2NC1 (Fig. 2H-J), ODF2NC2 (Fig. 2K-M) and ODF2N2C fusions (Fig. 2N-P; arrows in H,K,N) which showed also colocalisation to microtubules. In addition, all ODF2 fusion proteins were concentrated at the MTOC (microtubule organisation centre) (arrowheads in Fig. 2) which was also identified by anti- γ -tubulin staining (not shown). Moreover, association of ODF2N2C-GFP with microtubules was confirmed by its colocalisation to the mitotic spindle (Fig. 2Q-S, arrows). Our results suggest that ODF2 forms a fibrillar structure that is associated with the cytoskeleton. Moreover, treatment with nocodazole depolymerized intracellular MTs but did not disrupt the ODF2 network (Fig. 2T-V) suggesting that the cytoskeletal ODF2 network forms largely independently of microtubules.

Self-association of ODF2 proteins

Secondary structure predictions based on the amino acid sequence of ODF2 were performed with the program 'Coils' (Lupas et al., 1991) and indicated that ODF2 is an overall coiled-coil protein with the exception of the N-terminal region of about 50 amino acids (Fig. 3). Consistent with the predicted secondary structure, searches of sequence databases (Altschul et al., 1997) with the predicted ODF2 protein sequence

revealed that significant alignments were produced by proteins of the coiled-coil family, including myosins, lamins, tropomyosins and ODF3. In addition, ODF2 contains two leucine-zipper regions in the C-terminal half (Fig. 1A). In many proteins, the predicted coiled-coil segments lie in areas that are thought to play a functionally important role, for example, in mediation of oligomerization (Lupas et al., 1991).

In order to analyse in more detail the putative fibre forming capacity of ODF2 anticipated from transfection studies, we investigated whether ODF2 is able to interact with other molecules of ODF2 proteins and if specific parts of the ODF2 molecule may be necessary for interaction. ODF2-GFP fusion proteins were expressed in *cos-7* cells and the cell lysate mixed with a bacterial cell lysate containing ODF2-GST fusion proteins. GST fusion proteins and associated proteins were isolated on glutathione-Sepharose beads, separated on denaturing polyacrylamide gels (Laemmli, 1970) and GFP fusion proteins were detected by antibody incubation. The

presence of ODF2-GFP fusion proteins in the affinity purified fractions revealed that the full length ODF2 protein (ODF2NC) showed intermolecular interaction to full length ODF2 as well as to the N-terminal truncated ODF2 (ODF2N2C) and to the C-terminal truncated ODF2 (ODF2NC2) (Fig. 4A-C). Since ODF2NC2 does not contain any of the leucine-zipper regions (Fig. 1A) interaction seems to be independent of these regions and may be mediated solely by the overall coiled-coil structure of the protein. That the interaction does not depend on the leucine zipper regions was also shown by intermolecular interaction of the two C-terminal truncated proteins (ODF2NC2-GST and ODF2NC2-GFP) (Fig. 4E) which did not contain the leucine zipper regions. Intermolecular interactions were also demonstrated between NC1, which contains one of the two leucine zipper regions, and the N-terminal truncated protein N2C containing both leucine zipper regions (Fig. 4D), between the C-terminal truncated protein NC2 without leucine zipper regions, and the N-terminal truncated protein N2C (Fig. 4F), and between N-terminal truncated N2C-proteins (Fig. 4G). In control reactions without the addition of ODF2-GST proteins no ODF2-GFP-fusion proteins could be isolated, demonstrating that copurification of ODF2-GFP proteins is not based on interaction between GST and the full length ODF2 or between GST and GFP (data not shown).

To summarise, our results demonstrated the self-interacting capacity of ODF2 that is independent of any of the leucine zipper regions. Each part of the ODF2 molecule is able to interact with each other part of another ODF2 molecule.

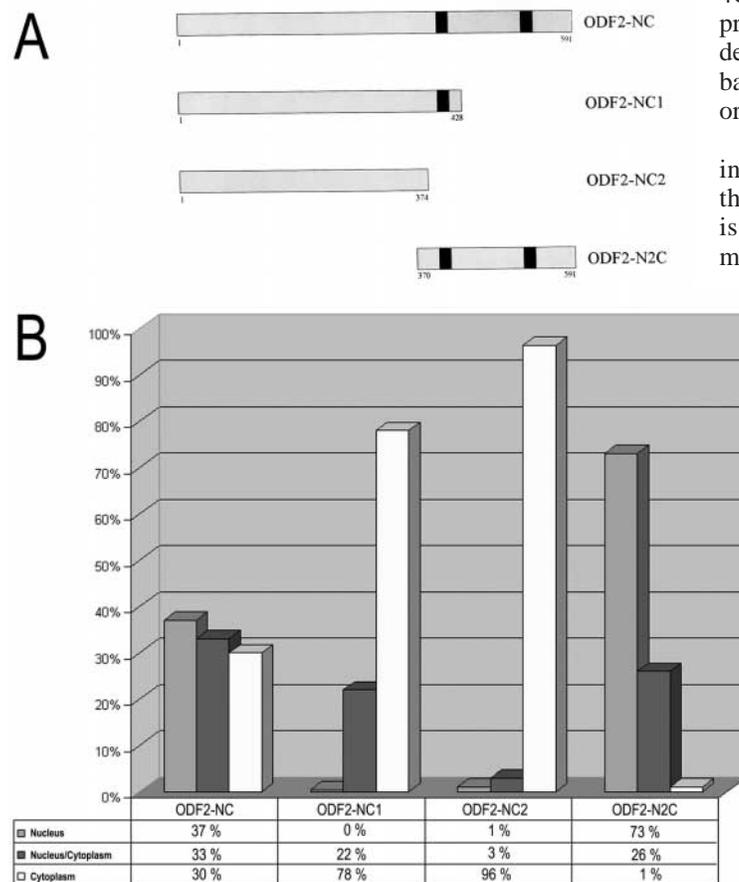


Fig. 1. Structural organization of the ODF2 protein and subcellular localization of ODF2-GFP fusion proteins. (A) Scheme of ODF2 fusion constructs. As outlined, different parts of ODF2 were fused to GFP at its C-terminal end, resulting in a C-terminal GFP tag, or to GST at its N-terminal end. The leucine-zipper regions are shown as black boxes in the C-terminal region of ODF2. (B) Cytoplasmic versus nuclear distribution of ODF2-GFP fusion proteins expressed in *cos-7* cells. Full length protein, ODF2NC, was slightly higher in the nucleus. The C-terminally truncated ODF2 proteins fused to GFP (ODF2NC1 and ODF2NC2) were mostly located outside of the nucleus, whereas the N-terminally truncated protein, ODF2N2C, fused to GFP was predominantly located inside the nucleus.

ODF2 interacts with microtubules

A partial association of ODF2 with the microtubule network could be detected in *cos-7* cells transfected with ODF2-GFP constructs (Fig. 2). To investigate whether cytological colocalisation reflects a physical interaction we performed cosedimentation as well as copurification assays.

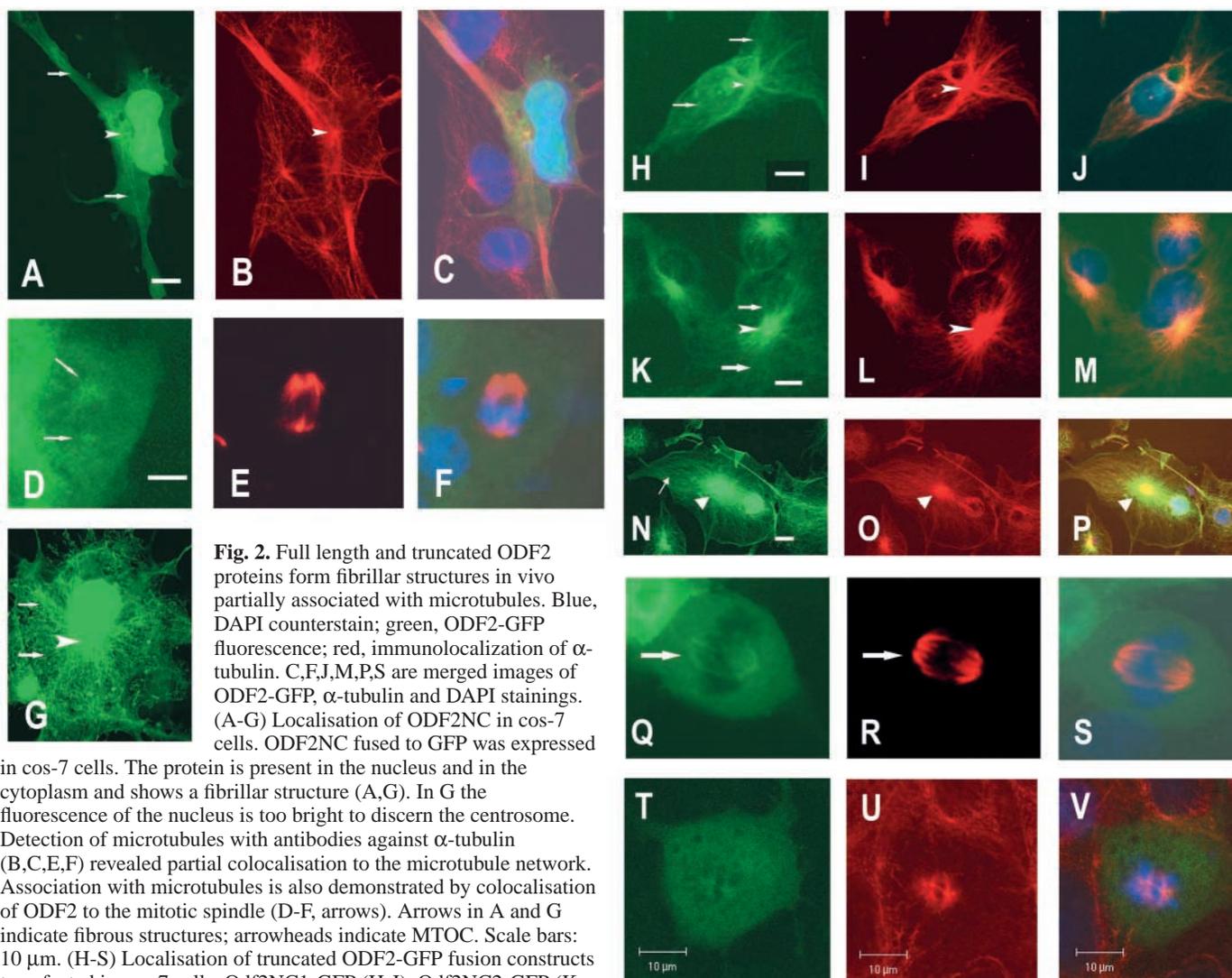
First, microtubules and microtubule-associated proteins were isolated from *cos-7* cells transfected with ODF2-GFP constructs using a modification of the centrifugation procedure described by Vallee and Collins (Vallee and Collins, 1986) and Kellogg et al. (Kellogg et al., 1989). ODF2 proteins were found to cosediment with microtubules since they are present in the final pellets after high speed centrifugation (Fig. 5A) in addition to tubulin (Fig. 5B). Moreover, sedimentation of ODF2 is microtubule-dependent (Fig. 5C). Under conditions in which microtubules are depolymerized, i.e. by incubation in the cold and without microtubule stabilising or assembly-promoting agents, no ODF2 proteins were found in the final pellet (Fig. 5C, lane 2) although ODF2-GFP proteins were present in the cells (Fig. 5C, lane 1). Cosedimentation of all four ODF2 constructs with microtubules therefore revealed that the microtubule association is independent of a specific region of the ODF2 molecule.

To verify the cosedimentation of ODF2 with microtubules, we investigated whether ODF2 is able to bind microtubules in *Xenopus* oocyte extracts by

performing GST pull down experiments. *Xenopus* oocyte extracts, supplemented with purified tubulin, were incubated with purified ODF2-GST fusion proteins under conditions that promote tubulin polymerization. The GST fusion proteins along with associated proteins were isolated by affinity purification on glutathione-Sepharose beads. Bound proteins were eluted from beads by the addition of glutathione and separated from insoluble material by centrifugation. The supernatant was aspirated and separated on a denaturing polyacrylamide gel, transferred to Hybond-C and probed with antibodies. Affinity isolation of ODF2-GST fusion proteins was verified by incubation of the western blot with anti-ODF2 antibodies (Fig. 6A). The full length ODF2-GST fusion protein (ODF2NC) as well as the C-terminally truncated fusion protein ODF2NC2-GST were present in the supernatant after elution with glutathione. α -tubulin could be detected in the same

fractions (Fig. 6B). A control experiment was performed under the same conditions but without the addition of purified ODF2-GST proteins. A very weak tubulin band was found after prolonged exposure time in the supernatant after elution with glutathione (not shown) whereas the result presented in Fig. 6B was found even after 2 minutes exposure. The tubulin-copurification assay therefore demonstrated an association of ODF2 with microtubules. Moreover, interaction is not dependent on the presence of the leucine zipper regions in the ODF2 protein since not only did the full length ODF2 protein show association with tubulin but association was also demonstrated for the C-terminally truncated ODF2 protein, ODF2NC2, which did not contain any leucine zipper regions.

Both the cosedimentation and the copurification assay therefore revealed an association of ODF2 with microtubules, and in addition demonstrated that the position and type of tag



in *cos-7* cells. The protein is present in the nucleus and in the cytoplasm and shows a fibrillar structure (A,G). In G the fluorescence of the nucleus is too bright to discern the centrosome. Detection of microtubules with antibodies against α -tubulin (B,C,E,F) revealed partial colocalisation to the microtubule network. Association with microtubules is also demonstrated by colocalisation of ODF2 to the mitotic spindle (D-F, arrows). Arrows in A and G indicate fibrous structures; arrowheads indicate MTOC. Scale bars: 10 μ m. (H-S) Localisation of truncated ODF2-GFP fusion constructs transfected in *cos-7* cells. Odf2NC1-GFP (H-J), Odf2NC2-GFP (K-M) and Odf2N2C-GFP (N-S) were transfected into *cos-7* cells and the microtubule network highlighted by anti- α -tubulin staining. ODF2NC1 and ODF2NC2 showed cytoplasmic localisation whereas ODF2N2C was preferentially located inside the nucleus. All constructs are found to be concentrated in the centrosome (arrowheads) and have a fibrillar appearance (arrows). Colocalisation to the mitotic spindle is demonstrated for ODF2N2C (Q-S). Scale bars: 10 μ m (H,K), 20 μ m (N). (T-V) Cytoplasmic organisation of ODF2 is mostly MT independent. Odf2NC-GFP transfected *cos-7* cells were treated with Nocodazol (2 μ g/ml for 1 hour) for disruption of microtubules. Microtubules were detected by anti- α -tubulin staining (U,V). Whereas Nocodazol treatment disrupted MT organisation (U), the cytoplasmic ODF2 organisation is not affected (T).

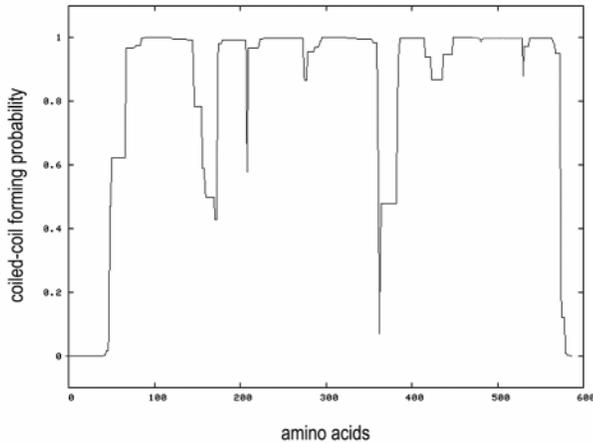


Fig. 3. ODF2 is a predicted overall coiled-coil protein. Secondary structure predictions for rat ODF2 were performed with the program 'Coils' (Lupas et al., 1991) with a scanning window of 28 residues. The coiled-coil forming probability demonstrates that ODF2 is a predicted overall coiled-coil protein with the exception of the N-terminal 50 amino acids.

do not influence the association of ODF2 with MTs. Instead of direct binding of ODF2 to tubulin association could be mediated by yet unknown proteins present in the cells. To discriminate between direct versus indirect interaction we performed a tubulin binding assay by incubation of purified ODF2-GST fusion proteins with purified tubulin in cell-free buffers. Binding assays were performed with all four ODF2-GST fusion constructs under two different conditions. Destabilising conditions which prevent microtubule formation were chosen to test if an interaction between ODF2 and tubulin at the molecular level may exist. Dependence of ODF2-tubulin interaction on microtubule formation was examined by using stabilising conditions, which promote microtubule formation. Successful isolation of ODF2 fusion proteins after incubation with tubulin was proved in all experiments. However, tubulin was not found to copurify with ODF2-GST constructs either under destabilising (not shown) or under stabilising conditions (Fig. 7), suggesting that association of ODF2 with microtubular tubulin is not direct and may instead be mediated via microtubule-associated proteins.

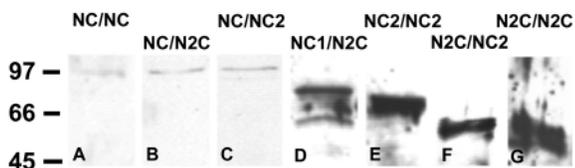


Fig. 4. Self-interaction of ODF2 does not depend on the presence of the leucine zipper regions. ODF2-GST fusion proteins were incubated with ODF2-GFP fusion proteins, and GST proteins as well as associated proteins were isolated by affinity chromatography on glutathione-Sepharose beads. Proteins were separated using denaturing SDS-PAGE and probed with antibodies against GFP. The ODF2 constructs used for each assay are shown above with the first construct designating the GFP fusion detected by anti-GFP antibodies, and the second construct designating the GST fusion.

Discussion

ODF2 was first described as a major protein of the outer dense fibres, which are very prominent and stable structures found in the sperm tails of animals with internal fecundation (Brohmann et al., 1997; Shao et al., 1997; Turner et al., 1997). In addition, ODF2 has also been described as a widespread component of the centrosome, the microtubule-organizing centre (MTOC) of the cell (Nakagawa et al., 2001). Based on the properties of known ODF proteins it was suggested that the ODF may form first by self-aggregation of fibre-forming proteins which are then stabilised by association of crosslinking proteins to build up the higher order fibre structure of ODF (Petersen et al., 2002). This view was also supported by a visible fibrillar structure on the surface of the ODFs (see Fawcett, 1975; Olson and Sammons, 1980).

Secondary structure predictions of ODF2 were performed with the program 'Coils' (Lupas et al., 1991) and indicated ODF2 as an overall coiled-coil protein. Since coiled-coils may play a functionally important role in mediating oligomerization we examined whether ODF2 is able to self-interact and to form fibres *in vivo*. Our data show that this is indeed the case. We demonstrated here that intermolecular interaction of ODF2 is independent on sequence motifs otherwise known to be involved in protein-protein interactions. Our results therefore suggest that the capacity of ODF2 molecules to interact with each other and to build up a fibrillar structure may depend mostly if not exclusively on its intrinsic coiled-coil structure.

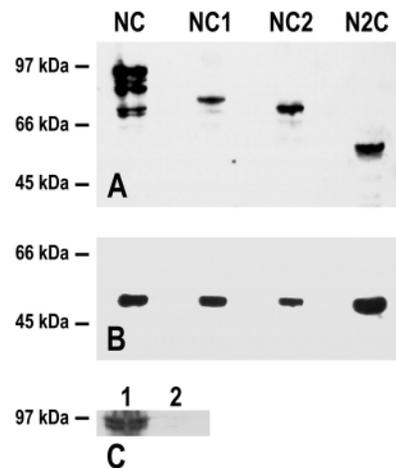


Fig. 5. Cosedimentation of ODF2 with microtubules. (A,B) Microtubules were isolated from cos-7 cells expressing one out of four ODF2-GFP fusion proteins under conditions that promote microtubule polymerization. Proteins were separated using denaturing SDS-PAGE and transferred to Hybond-C. Probing with antibodies against GFP (A) revealed cosedimentation of the full-length ODF2NC as well as the truncated ODF2 proteins ODF2NC1, ODF2NC2 and ODF2N2C with microtubules. (B) Detection of tubulin by anti- α -tubulin antibodies in the same fractions as in A. (C) Control performed with cos-7 cells expressing ODF2NC-GFP demonstrating the dependence of ODF2 sedimentation on microtubules. No ODF2 proteins were found in the final pellet if the experiment was performed under microtubule destabilising conditions (2), whereas the cellular debris after the first centrifugation step do contain ODF2-GFP (1). Incubation with antibodies against GFP.

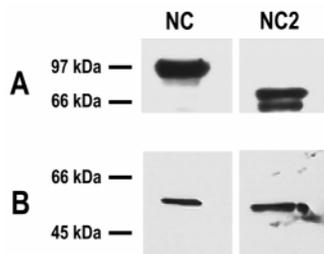


Fig. 6. Copurification of ODF2 with tubulin from *Xenopus* oocyte extracts. Purified ODF2NC-GST or ODF2NC2-GST proteins were incubated in *Xenopus* oocyte extracts supplemented with tubulin and under conditions that promote tubulin polymerization. GST fusion proteins and associated proteins were isolated by affinity chromatography, separated using denaturing SDS-PAGE, transferred to Hybond-C and probed with anti-ODF2 antibodies (A), and anti- α -tubulin antibodies (B).

Although ODF2 forms a fibrous network by overexpression in transfected *cos-7* cells this network in general seems to be intermediate in size between the microtubule and the microfilament network anticipating that ODF2 may belong to the diverge class of intermediate filament (IF) proteins. IF genes are differentially expressed in nearly all cells of the body. Although IF proteins in general constitute only a minor portion of the total cellular proteins, in some cells specific IFs are especially abundant (Fuchs and Weber, 1994; Fuchs and Cleveland, 1998). Most IF proteins are presently grouped into five different classes sharing only low sequence identities. Despite their diversity, IF proteins share a common secondary structure consisting of a central α -helical domain that is flanked by nonhelical amino-terminal and C-terminal domains, which vary in size and sequence. Assembly of IF proteins seems to be an intrinsic property and starts with the formation of dimers by parallel assembly of the α -helical domains forming an intertwined coiled-coil rod (Fuchs and Weber, 1994).

In many aspects, ODF2 resembles IF proteins. ODF2 is an abundant protein of spermatozoa in which it takes part in formation of the sperm tail-specific cytoskeleton. In most other somatic cells expression of *Odf2* is undetectable by

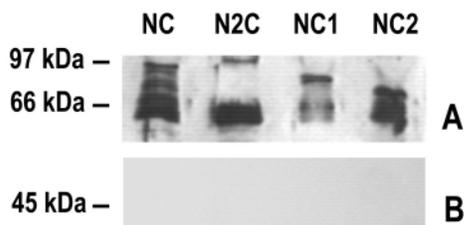


Fig. 7. ODF2 does not bind directly to tubulin. Purified ODF2-GST fusion proteins were incubated with purified tubulin under conditions that promote microtubule formation. GST fusion proteins were isolated by affinity chromatography, separated using denaturing SDS-PAGE, transferred to Hybond-C, and probed with anti-ODF2 antibodies (A), and anti- α -tubulin antibodies (B). Whereas ODF2 fusion proteins were successfully isolated, α -tubulin was not coprecipitated.

conventional methods (Hoyer-Fender et al., 1998) and even by RT-PCR (unpublished) although expression was demonstrated by EST sequencing. ODF2 is predicted to be a coiled-coil protein flanked by a short nonhelical amino-terminal end but without an apparent nonhelical C-terminal end (see Fig. 3). ODF2 assembles *in vivo* to form a fibrous anastomosed network within the cytoplasm. Assembly of ODF2 proteins seems to be an intrinsic property of the molecule, probably dependent on its coiled-coil structure. Since intermolecular ODF2 interaction seems not to reside on a specific sequence motif its fibre forming capacity most probably depends on its intrinsic coiled-coil structure although we could not totally exclude mediation by other proteins. ODF2 does not share sequence identities with known IF proteins, suggesting that ODF2 may not fit into any of the five major IF types or belong to any of the other known unconventional IF proteins. ODF2 does not even share sequence identities within the IF consensus sequences at the ends of the rod (Fuchs and Weber, 1994). Therefore, classification of ODF2 as an unconventional IF protein is not unequivocal although the properties of ODF2 as well as it being a major component of the highly insoluble ODF, which may function to resist stresses applied externally to the sperm tail, may indicate ODF2 is a novel IF protein.

The organization of the cytoskeletal IF network is also determined by associated non-IF proteins, which tether IF proteins to other cytoskeletal elements (Coulombe et al., 2000). Although ODF2 seems to be closely linked to microtubules as shown by partial colocalisation of the ODF2 network to the microtubule network in transfected *cos-7* cells as well as by its localisation to the appendages of the mother centriole (Nakagawa et al., 2001) and the close relationship of ODF formation to the tubulin doublets in the sperm tail (Escalier and David, 1984), we could not detect a direct interaction of ODF2 with tubulin. However, cosedimentation and copurification assays performed with cellular extracts demonstrated that ODF2 is associated with microtubules, suggesting that association may be mediated by other, as yet unknown, proteins present in the cell extract. In addition our experiments demonstrated that both the tubulin and the ODF2 networks exist almost independently of each other, as overexpression of ODF2 in *cos-7* cells has no obvious influence on the organisation of the tubulin network, and also disruption of the microtubule network did not disturb the ODF2 network. Our results further suggested that the formation of ODF in the sperm tail in close association with the axonemal microtubules, as well as the location of ODF2 to centrioles, may be mediated by proteins yet to be identified. One possible candidate is Hook 1, which interacts with ODF2 in a yeast two-hybrid assay (J. Neesen and B. Wellge, personal communication).

Overexpression of ODF2 in *cos-7* cells revealed that ODF2, besides being a cytoskeletal protein is also found in the nucleus. Although no nuclear localisation signal could be determined a corresponding signal has to be located in the C-terminal region of the truncated construct ODF2N2C. Preferential nuclear localisation of ODF2N2C could not be explained by diffusion, as GFP without any fusion is not preferentially located inside the nucleus. Nevertheless, it is possible that the preferential nuclear localisation of ODF2N2C in addition is dependent on a disturbed nuclear export as a result of the missing amino acid sequences. In contrast to ectopically expressed ODF2NC, the endogenous ODF2 protein

has not been found in the nucleus, however, this may be because of quantitative differences. Nevertheless, nuclear location has also been described for the centrosomal proteins ninein (Bouckson-Castaing et al., 1996) and TACC-proteins (Gergely et al., 2000) implying a potential nucleoskeletal function.

In the centrosome, ODF2 is a general component of the KI-insoluble scaffold (Nakagawa et al., 2001). The centrosome is the microtubule-organizing centre of the cell, which therefore plays a crucial role in organizing many processes in eukaryotic cells (Glover et al., 1993; Kellogg et al., 1994; Desai and Mitchison, 1997). Centrosomal microtubule nucleation requires recruitment and attachment of soluble γ -TURCs (γ -tubulin ring complexes) to the centrosome (Zimmerman et al., 1999; Schiebel, 2000), which could be facilitated by pericentrin (Dictenberg et al., 1998). Subsequent anchorage of γ -TURCs to the centrosome could be mediated by salt-insoluble centrosomal proteins of the centrosomal core, or 'centromatrix' (Moritz et al., 1998; Schnackenberg et al., 1998). Despite their importance little is known about how centrosomes interact with microtubules at the molecular level and in which functions centrosomal proteins are engaged. A number of centrosomal proteins have already been described. It seems that despite their divergent amino acid sequences most of them share a common secondary structure: they are coiled-coil proteins. Besides its structure, ODF2 [which shows over 90% sequence identities to cenexin (Lange and Gull, 1995)] showed also no sequence identity to known centrosomal proteins, such as centrosomin (Li et al., 1998), kendrin (Flory et al., 2000), ninein (Bouckson-Castaing et al., 1996), TACC-domain proteins (Gergely et al., 2000), or the PACT domain (Gillingham and Munro, 2000). This implies that centrosomal proteins may form a peculiar scaffold mediated mostly by their coiled-coil secondary structure and facilitating the anchorage of associated proteins or complexes. Despite no direct interaction between ODF2 and tubulin/microtubules could be shown, ODF2 binds to γ -tubulin (manuscript in preparation) implying that ODF2 may be involved in the recruitment or anchorage of γ -TURCs to the centrosome, which is a prerequisite to microtubule nucleation. Investigations of interactions between ODF2 and other known centrosomal proteins is still in progress in order to elucidate the centrosomal function of ODF2.

In conclusion, ODF2 is a self-interacting, microtubule-associated protein. However, microtubular association seems to be mediated by, as yet, unidentified proteins. ODF2 may belong to the IF proteins although it does not fall into one of the known classes of IF proteins. ODF2 is a centrosomal protein with peculiar functions in formation of the sperm tail cytoskeleton and possibly also in the nucleoskeleton and may be involved in the recruitment or anchorage of γ -TURCs to the centrosome. The features described here for ODF2, as having the ability to form a fibrillar structure inside the cell centred at the centrosome have already been described for the KI-insoluble centrosomal scaffold (Nakagawa et al., 2001) suggesting that ODF2 may be one important component of it.

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References

- Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W. and Lipman, D. J. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* **25**, 3389-3402.
- Baccetti, B., Pallini, V. and Burrini, A. G. (1973). The accessory fibres of the sperm tail. 1. Structure and chemical composition of the bull 'coarse fibres'. *J. Submicroscop. Cytol.* **5**, 237-256.
- Baltz, J. M., Williams, P. O. and Cone, R. A. (1990). Dense fibres protect mammalian sperm against damage. *Biol. Reprod.* **43**, 485-491.
- Bornens, M., Paintrand, M., Berges, J., Marty, M. C. and Karsenti, E. (1987). Structural and chemical characterization of isolated centrosomes. *Cell Motil. Cytoskeleton* **8**, 238-249.
- Bouckson-Castaing, V., Moudjou, M., Ferguson, D. J. P., Mucklow, S., Belkaid, Y., Milon, G. and Crocker, P. R. (1996). Molecular characterization of ninein, a new coiled-coil protein of the centrosome. *J. Cell Sci.* **109**, 179-190.
- Brohmann, H., Pinnecke, S. and Hoyer-Fender, S. (1997). Identification and characterization of new cDNAs encoding outer dense fibre proteins of rat sperm. *J. Biol. Chem.* **272**, 10327-10332.
- Burfeind, P. and Hoyer-Fender, S. (1991). Sequence and developmental expression of a mRNA encoding a putative protein of rat sperm outer dense fibres. *Dev. Biol.* **148**, 195-204.
- Coulombe, P. A., Bousquet, O., Ma, L., Yamada, S. and Wirtz, D. (2000). The 'ins' and 'outs' of intermediate filament organization. *Trends Cell Biol.* **10**, 420-428.
- Desai, A. and Mitchison, T. J. (1997). Microtubule polymerization dynamics. *Annu. Rev. Cell Dev. Biol.* **13**, 83-117.
- Dictenberg, J. B., Zimmerman, W., Sparks, C. A., Young, A., Vidair, C., Zheng, Y., Carrington, W., Fay, F. S. and Doxsey, S. J. (1998). Pericentrin and γ -tubulin form a protein complex and are organized into a novel lattice at the centrosome. *J. Cell Biol.* **141**, 163-174.
- Escalier, D. and David, G. (1984). Pathology of the cytoskeleton of the human sperm flagellum: axonemal and peri-axonemal anomalies. *Biol. Cell* **50**, 37-52.
- Fawcett, D. W. (1975). The mammalian spermatozoon. *Dev. Biol.* **11**, 391-436.
- Flory, M. R., Moser, M. J., Monnat, R. J. and Davis, T. N. (2000). Identification of a human centrosomal calmodulin-binding protein that shares homology with pericentrin. *Proc. Natl. Acad. Sci. USA* **97**, 5919-5923.
- Fuchs, E. and Cleveland, D. W. (1998). A structural scaffolding of intermediate filaments in health and disease. *Science* **279**, 514-519.
- Fuchs, E. and Weber, K. (1994). Intermediate filaments: structure, dynamics, function, and disease. *Annu. Rev. Biochem.* **63**, 345-382.
- Gastmann, O., Burfeind, P., Günther, E., Hameister, H., Szpirer, C. and Hoyer-Fender, S. (1993). Sequence, expression, and chromosomal assignment of a human sperm outer dense fibre gene. *Mol. Reprod. Dev.* **36**, 407-418.
- Gergely, F., Karlsson, C., Still, I., Cowell, J., Kilmartin, J. and Raff, J. W. (2000). The TACC domain identifies a family of centrosomal proteins that can interact with microtubules. *Proc. Natl. Acad. Sci. USA* **97**, 14352-14357.
- Gillingham, A. K. and Munro, S. (2000). The PACT domain, a conserved centrosomal targeting motif in the coiled-coil proteins AKAP450 and pericentrin. *EMBO Rep.* **1**, 524-529.
- Glover, D. M., Gonzalez, C. and Raff, J. W. (1993). The centrosome. *Sci. Am.* **268**, 62-68.
- Hoyer-Fender, S., Burfeind, P. and Hameister, H. (1995). Sequence of mouse *odf1* cDNA and its chromosomal localisation: extension of the linkage group between human chromosome 8 and mouse chromosome 15. *Cytogenet. Cell Genet.* **70**, 200-204.
- Hoyer-Fender, S., Petersen, C., Brohmann, H., Rhee, K. and Wolgemuth, D. J. (1998). Mouse *Odf2* cDNAs consist of evolutionary conserved as well as highly variable sequences and encode outer dense fibre proteins of the sperm tail. *Mol. Reprod. Dev.* **51**, 167-175.
- Kellogg, D. R., Field, C. M. and Alberts, B. M. (1989). Identification of microtubule-associated proteins in the centrosome, spindle, and kinetochore of the early *Drosophila* embryo. *J. Cell Biol.* **109**, 2977-2991.
- Kellogg, D. R., Moritz, M. and Alberts, B. M. (1994). The centrosome and cellular organization. *Annu. Rev. Biochem.* **63**, 639-674.
- Laemmli, U. K. (1970). Cleavage of structural proteins during assembly of the head of the bacteriophage T4. *Nature* **227**, 680-685.
- Lange, B. M. H. and Gull, K. (1995). A molecular marker for centriole maturation in the mammalian cell cycle. *J. Cell Biol.* **130**, 919-927.

- Li, K., Xu, E. Y., Cecil, J. K., Turner, F. R., Megraw, T. L. and Kaufman, T. C.** (1998). Drosophila centrosomin protein is required for male meiosis and assembly of the flagellar axoneme. *J. Cell Biol.* **141**, 455-467.
- Lindemann, C. B.** (1996). Functional significance of the outer dense fibres of mammalian sperm examined by computer simulation with the geometric clutch model. *Cell. Motil. Cytoskel.* **34**, 258-270.
- Lupas, A., van Dyke, M. and Stock, J.** (1991). Predicting coiled coils from protein sequences. *Science* **252**, 1162-1164.
- Moritz, M., Zheng, Y., Alberts, B. M. and Oegema, K.** (1998). Recruitment of the γ -tubulin ring complex to Drosophila salt-stripped centrosome scaffolds. *J. Cell Biol.* **142**, 775-786.
- Mortimer, S. T.** (1997). A critical review of the physiological importance and analysis of sperm movement in mammals. *Hum. Reprod. Update* **3**, 403-439.
- Murray, A. W. and Kirschner, M. W.** (1989). Cyclin synthesis drives the early embryonic cell cycle. *Nature* **339**, 275-280.
- Nakagawa, Y., Yamane, Y., Okanoue, T., Tsukita, S. and Tsukita, S.** (2001). Outer dense fibre 2 is a widespread centrosome scaffold component preferentially associated with mother centrioles: its identification from isolated centrosomes. *Mol. Biol. Cell* **12**, 1687-1697.
- Oko, R.** (1988). Comparative analysis of proteins from the fibrous sheath and outer dense fibres of rat spermatozoa. *Biol. Reprod.* **39**, 69-182.
- Olson, G. E. and Sammons, D. W.** (1980). Structural chemistry of outer dense fibres of rat sperm. *Biol. Reprod.* **22**, 319-332.
- Petersen, C., Füzesi, L. and Hoyer-Fender, S.** (1999). Outer dense fibre proteins from human sperm tail: molecular cloning and expression analyses of two cDNA transcripts encoding proteins of ~70 kDa. *Molec. Human Reprod.* **5**, 627-635.
- Petersen, C., Aumüller, G., Bahrami, M. and Hoyer-Fender, S.** (2002). Molecular cloning of Odf3 encoding a novel coiled-coil protein of sperm tail outer dense fibres. *Mol. Reprod. Dev.* **61**, 102-112.
- Sawin, K. E. and Mitchison, T. J.** (1991). Mitotic spindle assembly by two different pathways in vitro. *J. Cell Biol.* **112**, 925-940.
- Schalles, U., Shao, X., van der Hoorn, F. A. and Oko, R.** (1998). Developmental expression of the 84-kDa ODF sperm protein: localization to both the cortex and medulla of outer dense fibres and to the connecting piece. *Dev. Biol.* **199**, 250-260.
- Schiebel, E.** (2000). γ -tubulin complexes: binding to the centrosome, regulation, and microtubule nucleation. *Curr. Opin. Cell Biol.* **12**, 113-118.
- Schnackenberg, B. J., Khodjakov, V., Rieder, C. L. and Palazzo, R. E.** (1998). The disassembly and reassembly of functional centrosomes in vitro. *Proc. Natl. Acad. Sci. USA* **95**, 9295-9300.
- Shao, X., Tarnasky, H. A., Schalles, U., Oko, R. and van der Hoorn, F.** (1997). Interactional cloning of the 84-kDa major outer dense fibre protein Odf84. Leucine zippers mediate associations of Odf84 and Odf27. *J. Biol. Chem.* **272**, 6105-6113.
- Stearns, T. and Kirschner, M.** (1994). In vitro reconstitution of centrosome assembly and function: the central role of γ -tubulin. *Cell* **76**, 623-637.
- Stearns, T. and Winey, M.** (1997). The cell center at 100. *Cell* **91**, 303-309.
- Studier, F. W. and Moffat, B. A.** (1986). Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. *J. Mol. Biol.* **189**, 113-130.
- Towbin, H., Staehelin, T. and Gordon, J.** (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA* **76**, 4350-4354.
- Turner, K. J., Sharpe, R. M., Gaughan, J., Millar, M. R., Foster, P. M. D. and Saunders, P. T. K.** (1997). Expression cloning of a rat testicular transcript abundant in germ cells, which contains two leucine zipper motifs. *Biol. Reprod.* **57**, 1223-1232.
- Vallee, R. B. and Collins, C. A.** (1986). Purification of microtubules and microtubule-associated proteins from sea urchin eggs and cultured mammalian cells using taxol, and use of exogenous taxol-stabilized brain microtubules for purifying microtubule-associated proteins. *Methods Enzymol.* **134**, 116-127.
- Van der Hoff, M. J. B., Moorman, A. F. M. and Lamers, W. H.** (1992). Electroporation in 'intracellular' buffer increases cell survival. *Nucleic Acids Res.* **20**, 2902-2908.
- Vera, J. E., Brito, M., Zuvic, T. and Burzio, L. O.** (1984). Polypeptide composition of rat sperm outer dense fibres. A simple procedure to isolate the fibrillar complex. *J. Biol. Chem.* **259**, 5970-5977.
- Zimmerman, W., Sparks, C. A. and Doxsey, S. J.** (1999). Amorphous no longer: the centrosome comes into focus. *Curr. Opin. Cell Biol.* **11**, 122-128.