Cellular and Biochemical Analysis of an Outer Dense Fiber Protein 2 (Odf2) Variant and the Endogenous Odf2 / Cenexin in Functional Approaches.

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D7

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Der Mumi und dem Papili

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1. Kurzdarstellung

Der Zellzyklus somatischer Zellen ist ein komplexer Mechanismus zur Weitergabe des genetischen Materials, in dem das Centrosom und das Zytoskelett der Zellen zentrale Funktionen übernehmen. Er steuert die Duplikation des genetischen Materials und die anschließende Teilung der Zellen. Fehler, die während der Zyto – und Karyokinese auftreten, wie beispielsweise die Ausbildung multipolarer Spindeln, resultieren nicht selten in fehlerhafter Chromosomensegregation, Aneuploidien und unkontrolliertem Zellwachstum. Die Ausbildung mono- oder multipolarer Spindeln ist häufig assoziiert mit Deregulationen der Centrosomenduplikation (Badano et al., 2005; Marx, 2001; Nigg, 2002; Wang et al., 2004b).
Das Centrosom wird in tierischen Zellen von zwei orthogonal zueinander angeordneten Centriolen gebildet. Beide Centriolen sind über ein Proteinnetzwerk, der pericentriolären Matrix, miteinander assoziiert. Sie unterscheiden sich aufgrund ihrer centriolären Substrukturen und ihrer Proteinausstattung und lassen sich anhand dieser als Mutter – und Tochtercentriol charakterisieren (Bornens et al., 1987).
Das Outer dense fiber protein 2 (Odf2) wurde hauptsächlich assoziiert mit den distalen und subdistalen Anhängen des Muttercentriols, sowie der fibrillären Struktur der pericentriolären Matrix beschrieben (Nakagawa et al., 2001). Odf2 scheint ein wichtiges, wenn nicht notwendiges, Protein zu sein, das in den Zellzyklus, der korrekten Centrosomenreifung (Lange and Gull, 1995), der Mikrotubulinukleation und letztendlich auch in der fehlerfreien Chromosomensegregation involviert zu sein scheint (Soung et al., 2006).
Diese Arbeit dokumentiert die Identifikation von somatischen Splicevarianten von Odf2 und zeigt, dass Odf2- und Cenexintranskripte differentielle Splicevarianten eines Gens sind (Huber and Hoyer-Fender, 2007). Es wurde gezeigt, dass der N-terminale Bereich sowohl für die centrosomale Lokalisation des Proteins als auch für dessen Assoziation mit dem Basalkörper primärer Cilien notwendig ist (Huber et al., 2008). Eine funktionelle Charakterisierung erfolgte mittels biochemischer und zellbiologischer Methoden. Um Odf2 weiter zu charakterisieren wurden Interaktionsstudien durchgeführt. Eine direkte Interaktion mit der nicht phosphorylierten Form des Retinoblastoma Proteins konnte gezeigt werden ebenso wie eine Assoziation mit dem Kinetochorprotein Zwint-1.
Diese Daten zeigen erstmals einen Zusammenhang zwischen dem centrosomalen Protein Odf2, Histonmodifikationen und Chromatindynamik, die als essentiell für die korrekte Verteilung der Chromosomen beschrieben wurden, und der Separierung der Chromosomen auf.

1. Summary

The genome of a somatic cell is duplicated once every cell - cycle. The centrosome as well as the cytoskeleton plays central roles in orchestrating cell cycle progression and its proper function. The duplication of the genetic material and cytokinesis are guided by the cell - cycle. Errors, like the assembly of mono – or multipolar spindles may occur during cyto – or karyokinesis and often result in chromosome missegregation, aneuploidy and aberrant cell proliferation. Formation of mono-, or multipolar spindles are often associates with the deregulation of centrosome duplication (Badano et al., 2005; Marx, 2001; Nigg, 2002; Wang et al., 2004b).

In mammalian cells the centrosome is composed of a pair of barrel-shaped centrioles. Pericentriolar material surrounds the centrosome and forms a fibrous centriole connecting structure. The centrioles differ in their substructures and protein composition and can be characterized as mother and daughter centriole (Bornens et al., 1987).

The Outer dense fiber protein 2 (Odf2) is a component of the centrosome. It localizes to the distal and subdistal appendages of the mature centriole, and the fibrous structures of the pericentriolar matrix (Nakagawa et al., 2001). Odf2 seems to be an important, unless essential protein involved in orchestrating the cell-cycle, centrosomal maturation (Lange and Gull, 1995), microtubule nucleation and correct chromosome segregation (Soung et al., 2006).

In this study somatic splice variants of Odf2 / Cenexin have been isolated. Odf2 and Cenexin transcripts are the products of differential splicing of a single gene (Huber and Hoyer-Fender, 2007). The identified variant posses a N-terminal extension necessary for centrosomal localization and association with the basal body of primary cilia (Huber et al., 2008).

In this study, the functional role of Odf2 / Cenexin has been dissected by cell biological and biochemical analysis. A direct interaction between Odf2 / Cenexin and the non-phosphorylated form of the retinoblastoma protein has been shown. Furthermore it was possible to show an association with the kinetochore protein Zwint-1.

These results suggest a connection between the centrosomal protein Odf2 / Cenexin and histone modification, chromatin dynamics, and chromosome segregation, and therefore a possible connection to aneuploidy and cancer.

2. Introduction

2.1. The Cell Cycle

The duplication of the genetic material and the following division of eukaryotic cells transmits the genetic information from one cell generation to the next. This complete process needs to be tightly controlled by many different factors. The cell reproduction commences with the duplication of the cell's components, including the duplication of each chromosome in synthesis phase (S phase). It goes on with the distribution of the duplicated cell components into two daughter cells (mitosis), and ends with cytokinesis. The progress of these cell-cycle events is regulated and coordinated by cyclins, cyclin-dependent kinases (cdks) and phosphatases.

The S-phase is characterized by the replication of the DNA and chromosome duplication. This chromosome duplication requires increased synthesis of proteins, for example histones and because of this is called synthesis-phase. After successful S-phase cells enter a gap phase, named G2. In this phase the duplicated and synthesized material will be controlled prior to progression to the next phase. Gap phases are important regulatory transition points of cell cycle progression. Following G2 cells enter mitosis. The pair of sister chromatids will be pulled apart by the mitotic spindle to opposite poles of the dividing cell and the two sets of chromosomes are packed into daughter nuclei. After cytokinesis, in which the cell has divided, another gap phase follows. In G1 due to environmental stimulation the cell decides to undergo another division cycle or exit the cell cycle – entering G0.

2.2. Cell Cycle Control by Cyclin-Cdk complexes

Cell cycle progression and therefore the transition from one cell-cycle phase to another is controlled by a complex regulatory network. The main components that coordinate the order and timing of cell cycle events are cyclin-dependent kinases (cdks). Cdks are activated by binding to regulatory proteins, the cyclins, which are expressed and degraded at different cell cycle stages (Fig.1). Once activated cdks are able to catalyze the covalent attachment of phosphate groups to their substrates, followed by changes in the activation state of the target proteins controlling cell cycle processes.

The cell will enter the cell cycle under optimal growth conditions for cell proliferation. The G1 cyclin – cdk complex (cyclin D – cdk4/6) is expressed and activates transcription factors that promote transcription of genes encoding enzymes required for DNA synthesis as well as genes encoding S-phase cyclin and cdks (Blain, 2008). Numerous gene products are involved in G1/S transition but best understood are those whose expression is controlled by

E2F transcription factors (Cam and Dynlacht, 2003). E2Fs can be classified as activators or repressors of cell cycle checkpoints and interact with members of the retinoblastoma (Rb) pocket protein family controlling S – phase genes (van den Heuvel and Dyson, 2008).

The S cyclin – cdk complex (cyclin E/A – cdk2) is inactivated by S phase inhibitors until the G1 cyclin – cdk induces degradation of the inhibiting complex through phosphorylation. The active S cyclin – cdk (cyclin A – cdk2) phosphorylates regulatory sites in proteins forming pre-replication complexes and other early cell-cycle events (Koff et al., 1992; Tsai et al., 1993). During S - phase and G2 mitotic cyclin - cdk complexes are synthesized. However their activity is inhibited by phosphorylation at regulatory sites of the cdk subunit until DNA synthesis is completed. At the onset of mitosis, all M - cdks (cyclin A/B – cdk1) are activated by dephosphorylation and the G2/M checkpoint is passed (Ubersax et al., 2003). The activated M cyclin – cdk complex phosphorylates and therefore activates multiple proteins that promote chromosome condensation, retraction of the nuclear envelope, assembly of the mitotic spindle, and alignment of condensed chromosomes in metaphase plate (Morgan, 1997).

During mitosis the anaphase promoting complex (APC) marks key regulatory proteins for proteasomal degradation by polyubiquitination (Peters, 2002). This degradation of proteins leads to inactivation of protein kinase activity of the mitotic cdks. The decrease of mitotic cdk activity in turn leads to chromosome decondensation, nuclear envelope re-forming, and cytokinesis.

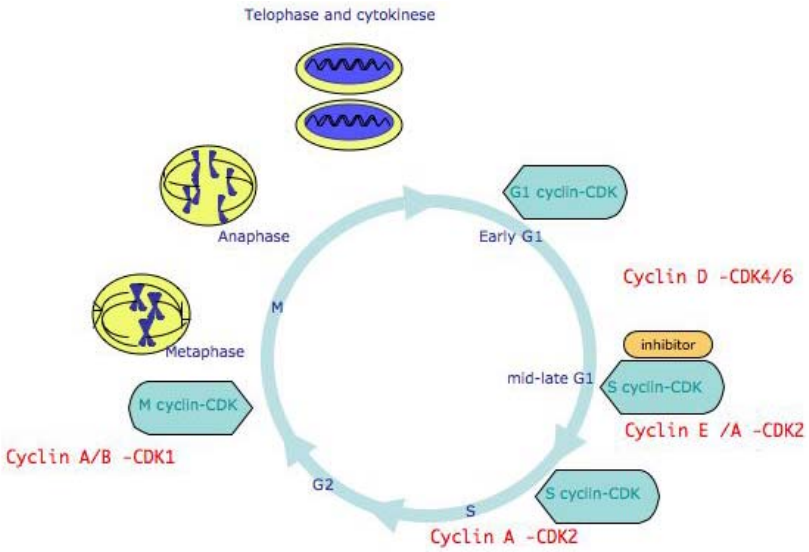

Figure 1: Cell Cycle Control by cyclin-cdk complexes. Cyclins are cdk regulatory proteins and are expressed at different stages of the cell cycle. Activated cdk's are able to phosphorylate target proteins important for entering the following cell cycle phase and therefore cell cycle progression.

2.3. The Role of Chromatin and its Regulation

The genetic material inside the nuclei of every eukaryotic cell exists in form of chromatin, macromolecular complexes of nucleic acids and proteins. The DNA is replicated during S-phase. This process is tightly controlled to occur only once per cell cycle. Along with the duplication of the DNA the associated proteins are also duplicated and loaded onto the newly synthesized DNA. The distinct epigenetic marks like DNA methylation (imprinting) or posttranslational modifications of histone proteins are replication overlapping inherited. Chromatin is a highly dynamic structure. The degree of condensation and therefore the accessibility of the underlying DNA is regulated by multi enzyme complexes that control gene expression, DNA replication and repair.

The basic unit of chromatin is the nucleosome (Fig.2). 147bp of DNA are wrapped in ~1,7 helical turns around a histone octamer. The octamer is composed of eight basic histone proteins: H2A, H2B, H3 and H4 (two copies each) (Kornberg, 1974; Kornberg and Thomas, 1974).

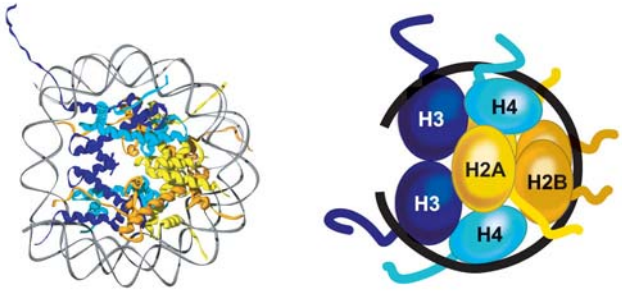

Figure 2: Nucleosome Structure. Each Nucleosome is build up by two H2A, H2B, H3, and H4 histone proteins. Around this octamer the DNA is turned. Clearly displayed are the histone tails, which extended from the compact octamer. These are targets of many modifications like methylation, phosphorylation, or acetylation (Allis, 2007).

The histone octamers are arranged along the DNA like beads on a string and can be captured by the linker histone H1, which binds the DNA at its entry/exit point on the surface of the octamer (Bates and Thomas, 1981; Thomas, 1999). Out of this compact structure the unstructured histone tails extend from the surface of the nucleosome. The tails are substrates of enzymes that add posttranslational modifications. These modifications alter the biophysical characteristics of the nucleosome and either act in cis through intranucleosomal combinatorial interactions between single modifications or in trans through the recruitment of effector proteins that can mediate internucleosomal contacts. Histone modifications include methylation, acetylation, phosphorylation, ubiquitinylation, poly ADP-ribosylation and sumoylation (see Fig.3; (Allfrey, 1966; Johansen and Johansen, 2006). The combinatorial existence of different modifications lead to the postulation of the so called “histone code”.

Different combinations of histone modifications lead to distinct downstream effects (Strahl and Allis, 2000).

Histone 3 lysine 9 trimethylation (H3K9me3) is considered a marker for pericentric heterochromatin (Jenuwein and Allis, 2001; Peters et al., 2002), regions where the nucleosomes are tightly packed into higher order chromatin structures. Less condensed, more open structures are named euchromatin. These regions can be characterized as gene rich. Transcription of some euchromatic genes seem to be associated with histone 3 serine 10 phosphorylation (H3S10ph). Additionally H3S10ph is involved in chromosome condensation at the onset of mitosis (Johansen and Johansen, 2006).

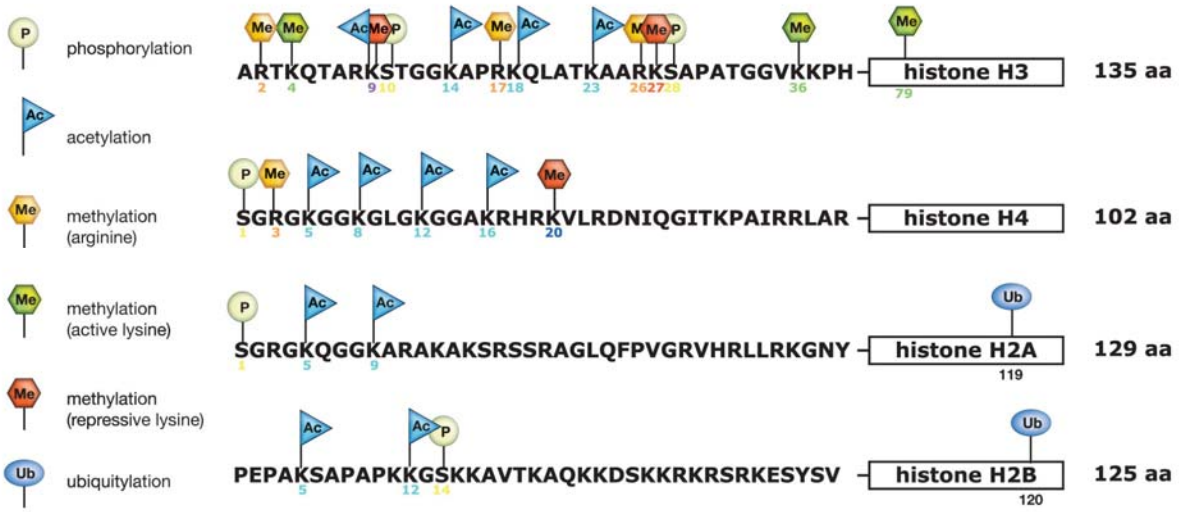

Figure 3: Modification of Histone Tails. The histones forming the nucleosome are characterized by a lot modifications on their tails. Acetylation, phosphorylation, methylation, and ubiquitylation are described and are characteristic for chromatin arrangements (Allis, 2007).

2.4. Mitosis

Mitosis, the process in which sister chromatids are separated and cytokinesis happens is divided into five steps: prophase, metaphase, anaphase, telophase, and cytokinesis (Fig.4).

Prophase begins at the onset of chromosome condensation, goes on with the separation of the centrosomes, and the initiation of spindle assembly. The metaphase is characterized by the nuclear envelope breakdown and complete attachment of sister chromatids to the mitotic spindle followed by their alignment in the metaphase plate. In anaphase the sister chromatids are separated and pulled to the opposite poles of the spindle. Telophase completes mitosis. Repackaging of chromosomes and nuclear components into the newly formed nuclei of the daughter cells occur.

The entry of mitosis is mediated by cdks (e.g. cdk1) and in addition by polo-like kinase (Plk) and Aurora kinases. Plk gets activated during G2/M transition and is required for the proper

timing of the mitotic entry and for the assembly of a proper bipolar spindle. Aurora A, a member of the Aurora family of kinases is involved in centrosome function and spindle assembly (Marumoto et al., 2005). Aurora B is proposed to regulate kinetochore assembly and bipolar chromosome attachment, the spindle checkpoint, chromosome condensation and cohesion, and the coordination of chromosome segregation and cytokinesis (Shannon and Salmon, 2002). Sister chromatids synthesized in S-phase remain physically connected by the protein complex cohesin until their timely segregation. The removal of centromeric cohesin, promoted by Plk1 and Aurora B, is a central event in sister chromatid separation at the metaphase-anaphase transition.

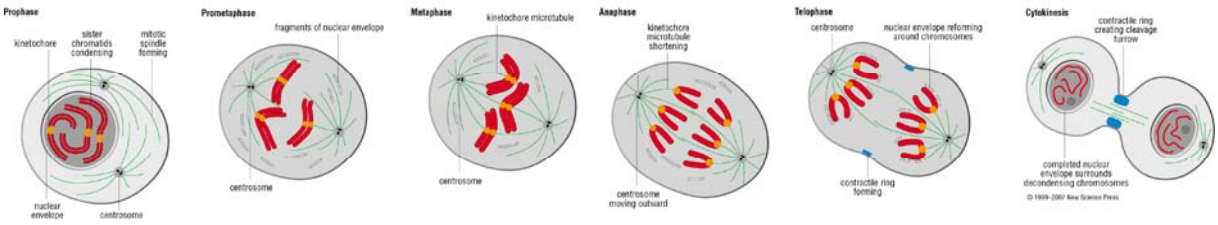

Figure 4: Stages of Mitosis. Mitosis is divided into five steps: prophase, metaphase, anaphase, telophase, and cytokinesis. In prophase the sister chromatids are condensing and the formation of the mitotic spindle begins. In metaphase the nuclear envelope breaks down and chromatids are aligned in metaphase plate. In anaphase the chromatids are pulled to the spindle poles. Division of the cell commences in telophase also the reformation of the nuclear envelope. Mitosis will be completed by cytokinesis, when the daughter cells separate (Morgan, 2006).

2.5. From Centromeres to Centrosomes

The prophase of mitosis is characterized by the assembly of the bipolar mitotic spindle and is completed when microtubules (MTs) from both spindle poles are attached to each sister chromatid pair. A kinetochore multiprotein complex on its centromere region mediates each chromatid spindle attachment. This attachment stabilizes the astral microtubules and establishes the bipolar character of the mitotic spindle (Kirschner and Mitchison, 1986). In metaphase three classes of microtubules are found (Fig.5): (i) kinetochore microtubules, (ii) interpolar microtubules, which link both spindle poles with each other in the midzone of the spindle, and (iii) astral microtubules which extend from the spindle poles into the distal lumen, anchor and position the spindle in the cell (Varmark, 2004). After removal of cohesin, sister chromatids are pulled apart to the spindle poles, the so - called centrosomes.

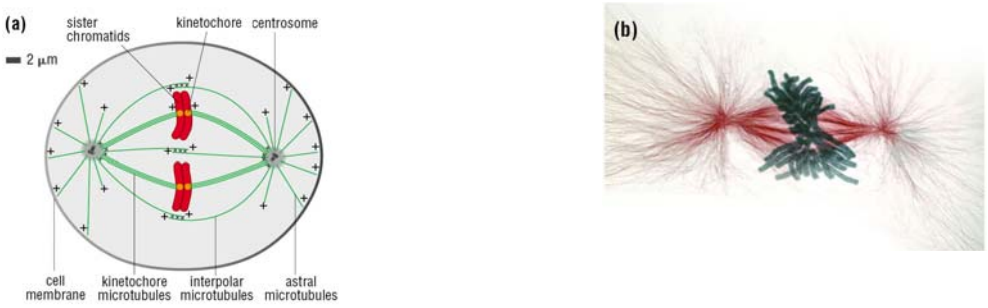

Figure 5: Microtubule Organization in a Mitotic Cell. In a mitotic spindle three classes of microtubule are found. Astral, kinetochore, and interpolar microtubules. All microtubules are nucleated from the centrosomes, and are plus end directed into the cell surface (Morgan, 2006).

In post-mitotic cells the centrosome consists of two centrioles surrounded by electron-dense pericentriolar material named pericentriolar matrix (PCM). The PCM forms a fibrous centriole connecting structure where nucleation and anchoring of microtubules by γ-tubulin ring complexes (γ-TuRC) take place (Bornens et al., 1987). In addition to the PCM the mature centriole is able to anchor MTs on its subdistal appendages (Piel et al., 2000). The centrioles each are built up of nine microtubule triplets, arranged to form a cylindrical structure. The mature centriole is characterized by nine distal (docking at the plasma membrane) and nine subdistal appendages (i.e. MT anchoring) (Paintrand et al., 1992) that are formed by characteristic proteins like Odf2 / Cenexin (Lange and Gull, 1995; Nakagawa et al., 2001).

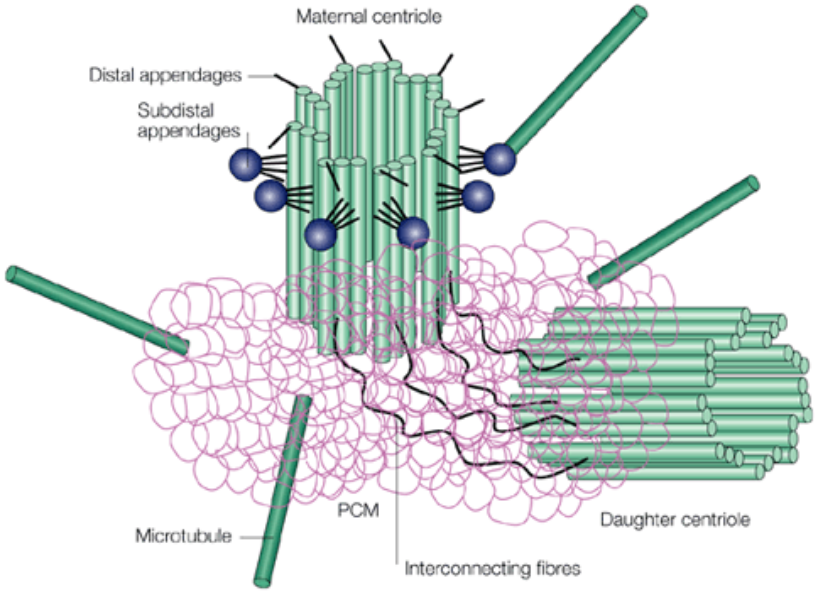

Figure 6: Centrosome composition. The centrosome of animal cells is normally is built up of two connected centrioles surrounded by the pericentriolar matrix (PCM). The PCM acts as a matrix for anchoring microtubules by γTuRCs. Nine MT triplets form each centriole. The mature centriole, also called mother centriole is characterized by distal and subdistal appendages formed by typical proteins (Doxsey, 2001).

During the cell cycle each centriole is duplicated in a semi-conservative manner. Each daughter cell will get a pair of centrioles that will form the centrosome of the cell and act during interphase as the major microtubule organization centre (Kochanski and Borisy, 1990).

During S-phase not only the DNA is replicated but also the duplication of the centrosome starts with the initiation of procentriole assembly. First centrin is recruited to the procentriole assembly sites of the mature and daughter centriole (Bornens, Kodjakov, 2008, personal communication) Next centriole microtubules are assembled and on the proximal tip a so - called cartwheel structure is formed. This process is followed by procentriole elongation in G2. In late G2 the maturation of the centrioles starts: appendages proteins and γ-tubulin ring complexes (γTuRCs) are recruited, and centrosome separation begins by disassembly of the fibrous linker of the two pairs of centrioles (but not of the two centrioles forming one centrosome) (Stearns, 2008, personal communication). The new built centrosomes are separated and under influence of Plk1 and Aurora A spindle pole components are recruited (Blagden and Glover, 2003). The centrosomes act as spindle pole bodies during mitosis. Interestingly the appendages are no longer visible until cytokinesis and are newly formed during cytokinesis / G1 transition. In G1 the centrosome is composed like described in Figure 6 and acts as major microtubule organization centre that orchestrates the astral MT that participate in various cellular functions like cell motility, cell polarity, cell adhesion, and intracellular trafficking. It is well known that disorientation, or disengagement occurs during early G1 phase before completion of cytokinesis (Piel et al., 2001). This corresponds to the loss of orthogonal association between the mature and daughter centrioles due to Separase protease activity which also drives the separation of sister chromatids prior to anaphase (Tsou and Stearns, 2006).

3. Results

Each chapter within the results starts with a one-site description of the aim of the particular manuscript in context of the complete thesis, the status of the manuscript, and optional the authors and their contribution to the practical work.

3.1. Alternative splicing of exon 3b gives rise to ODF2 and Cenexin.

In this part, the isolation of an Odf2 isoform is described that shares sequence homology to Cenexin. Sequence analysis has shown that Odf2 and Cenexin are alternative splice variants of the same gene. Therefore the relationship between Odf2 and Cenexin was demonstrated.

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Alternative splicing of exon 3b gives rise to ODF2 and Cenexin

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Abstract. ODF2 was first identified as the major component of the sperm tail outer dense fibers. Additionally, ODF2 is a critical component of the mature centriole of the animal centrosome where it locates to the distal appendages. Moreover, generation of primary cilia strictly depends on ODF2. The mature centriole is characterized further by recruitment of Cenexin. Albeit highly similar in sequence the relationship between ODF2 and Cenexin has not been investigated. We demonstrate here that ODF2 and Cenexin are alternative splice products by identifying a novel exon 3b encoding Cenexin specific amino acids. Even though ODF2 is the main isoform in testicular tissue RT-PCR analyses revealed that isoforms are not restricted to specific tissues.

ODF2 is the major protein component of the outer dense fibers (ODF) of the mammalian sperm tail. The outer dense fibers are accessory fibers accompanying the axonemal tubuli doublets on their outer site. These fibers are composed of more than a dozen different proteins (Vera et al., 1984; Oko, 1988; Petersen et al., 1999) of which only a few have been identified. Although not involved in active motility they seem to be important for the stability and the elastic recoil of the sperm tail as well as for support of the flagellar beat (Baltz et al., 1990; Lindemann, 1996). Impaired development of the outer dense fibers has been described as a major cause of tail abnormalities in infertile men (Haidl et al., 1991) indicating an important function in sperm motility and/or morphology. *Odf2* cDNAs have first been isolated from rat, mouse and human testis libraries and comprise a lot of splice isoforms (Brohmann et al., 1997; Shao et al., 1997; Turner et al., 1997; Hoyer-Fender et al., 1998; Petersen et al., 1999). *Odf2* transcripts are highly abundant in postmeiotic male germ cells (Brohmann et al., 1997; Turner et al., 1997; Hoyer-Fender et al., 1998; Schalles et al., 1998; Petersen et al., 1999) but are not restricted to them. Expression analyses by RT-PCR and EST-Blast revealed transcription of *Odf2* in a lot of somatic tissues including heart and brain, as well as in carcinoma cell lines (Hoyer-Fender et al., 2003). Supportingly, ODF2 has been identified as a microtubule-associated protein that is an intrinsic component of the centrosome in animal species (Nakagawa et al., 2001; Hoyer-Fender et al., 2003; Donkor et al., 2004).

The centrosome is located at the cell center at the vicinity of the cell nucleus. It is the major microtubule organizing center of the eukaryotic cell (MTOC). During mitosis, the centrosome functions to generate the mitotic spindle with major impacts on chromosome segregation and cytokinesis. In addition, the centrosome seems to play an important role in orchestrating cell cycle progression (Kellogg et al., 1994; Stearns and Winey, 1997; Zimmermann et al., 1999; Bornens, 2002; Fry and Hames, 2004).

Each centrosome contains two unequal centrioles of which the older or mother centriole is characterized by the presence of distal and subdistal appendages. Among those proteins that specifically associate with the appendages of

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the mother centriole are ODF2 (Nakagawa et al., 2001) and Cenexin (Lange and Gull, 1995). ODF2-deficient mother centrioles lack their appendages and are unable to generate primary cilia (Ishikawa et al., 2005) pointing to the significance of ODF2 in cilia formation.

Although *Odf2* and *Cenexin* sequences are highly similar their relationship is unclear. We therefore asked whether both are alternative spliced isoforms restricted or preferentially found in testicular and somatic tissue, respectively. The 5′ ends, which are the most divergent between *Odf2* and *Cenexin* were isolated out of mouse NIH3'T3 cell lines using *Odf2* sequence specific primers. Our results show that Odf2/Cenexin are indeed alternatively spliced transcripts and that N-terminal sequences in Cenexin are derived from a novel exon 3b. Moreover, in somatic cells a further isoform exists consisting of Cenexin sequences extended more N-terminally with sequences found previously exclusively in testis derived ODF2. However, we could not find a tissue-specific restriction of these diverse splice isoforms albeit formerly described ODF2 sequences are more abundant in testis tissue.

Materials and methods

5′ RACE

NIH3'T3 cells (ATCC) were grown in DMEM, 10% fetal bovine serum, 5% penicillin/streptomycin (all Gibco) at 37°C in 5% CO_2. Total RNA was prepared using peqGOLD TriFast reagent (Peqlab, Germany) and precipitated with ethanol. Purification of polyA-RNA from total RNA was performed using Dynabeads Oligo $(dT)_{25}$ (Dynal Biotech, Oslo, Norway). 5′ ends were generated by RACE using BD Smart RACE cDNA Amplification Kit (BD Biosciences Clontech, Palo Alto, USA) and sequence specific primers (DH1rev: CAGCTTCCCGATGGTATCCTTCAAG or DH2rev: CGGAATCCTCTTTGCAGTGCGT), and PCR products cloned into pCR 2.1 (Invitrogen, Karlsruhe, Germany). DNA was prepared by alkaline lysis procedure (Birnboim and Doly, 1979) using QIAprep Spin Miniprep Kit (Qiagen, Hilden, Germany) and sequenced (Macrogene, Korea). Nucleotide and amino acid sequences were analysed using BLAST program (http://www.ncbi.nlm.nih.gov) or Multalin (http://www.toulouse.inra.fra/multalin.html).

RT-PCR

Total RNA from mouse tissues was prepared using peqGOLD TriFast reagent. cDNA was generated using oligo dT primer and AMV reverse transcriptase. Amplification of *Odf2* 5′ region was performed with primer pair 13.8 EcoRI for (GGAATTCATGTCCGCCTCATCCTCAGGC) and DH1rev. A nested PCR was performed on first PCR products with primers Insfor (CTTCAACTCCCCCCTTACATG) and DH1rev.

PCR products were isolated from agarose gels and sequenced (MWG Biotech, Martinsried).

Results

Odf2/Cenexin sequences comprise a lot of alternative spliced forms

The 5′ end of *Odf2* was amplified out of cDNA generated from NIH3'T3 mouse cells. RACE products were subcloned and sequenced in both directions. The nucleotide sequences of all clones are very similar to each other and demonstrate their close relation to *Odf2* (AF000968 and AF034105). However, there is a certain variability concerning the presence/absence of short stretches of nucleotides resulting in additional or missing amino acids (see supplementary Fig. 1, www.karger.com/doi: 10.1159/000109621).

The most divergent RACE clones DH9.5 (accession no. DQ091767) and DH13.8 (accession no. DQ091769) were compared to the nucleotide sequences of mouse *Odf2* cDNA clones AF000968 and AF034105 (Brohmann et al., 1997) isolated from testis, to cDNA clone BC057001 from mouse brain (Strausberg et al., 2002), to rat *Cenexin 2* cDNA (AF162756; http://www.ncbi.nlm.nih.gov), and to the genomic *Odf2* sequence (NT039206) (see Suppl. Fig. 1). Clones AF000968, Cenexin2, DH13.8, DH9.5, and BC057001 all start in exon 1 although they do not have identical start sequences. AF000968 starts at the most 5′ end but moreover is characterized by an internal deletion of 10 nt. Exon 2a is the transcriptional start exclusively of AF034105 (Hoyer-Fender et al., 2003). Sequences of exon 2a are not found in any other *Odf2* clone. Exon 2b is found in AF034105, BC057001 and DH13.8. In DH13.8 exon 2b is not completely identical to the genomic sequence which, however, most probably relies on incorrect sequencing results. Exon 3 is present in all clones but clone AF000968 has an additional internal deletion of 26 nt in this region. Exon 4 is also present in all clones but 57 nt of the 3′ region are deleted in AF034105 and DH9.5. From exon 5 on all sequences are nearly identical. Surprisingly, we found an additional exon (exon 3b) not described before (Hoyer-Fender et al., 2003) which is present in BC057001, Cenexin2, DH13.8 and DH9.5 (and in DH8.5 and DH11.7, see below) although DH13.8 showed a deletion of 37 nt in the 5′ region (see suppl. Fig. 1, www.karger.com/doi: 10.1159/000109621 for divergence of sequences).

The exon-intron organization of the 5′ region of the mouse *Odf2* gene (NT039206) and exon sequences present in *Odf2* isoforms isolated from mouse testis (AF000968 and AF034105; Brohmann et al., 1997) and from NIH3T3 cells (DH13.8; this manuscript) and in rat *Cenexin2* are schematically drawn in Fig. 1a. In exons 2b, 3b and 4 translational start codons are found, giving rise to divergent N-terminal ends (Fig. 1b). Although testis cDNA clone AF000968 comprises exons 1 and 3 the translational start is not until exon 4 giving rise to N-terminal amino acid sequence MKG. In the testis cDNA clone AF034105 exon 2a is the transcriptional start and exon 1 is skipped. The translational start is present in exon 2b giving rise to N-terminal amino acid sequence MSASS. Cenexin2 is similar to the testis derived clone AF000968 in comprising exons 1 and 3 and skipping exons 2a and 2b. However, Cenexin2 contains another exon (3b) including a further translational start which gives rise to N-terminal amino acid sequences MKDR. MKDR is also the N-terminal end of BC057001 (from mouse brain), and RACE clones DH8.5, 9.5 and 11.7 from NIH3T3 cells. The transcript of RACE clone DH13.8 includes exons 1, 2b, 3, and 3b whereas exon 2a is omitted. However, the translational start is in exon 2b resulting in N-terminal amino acid sequences found also in AAB87525.1 (AF034105) isolated

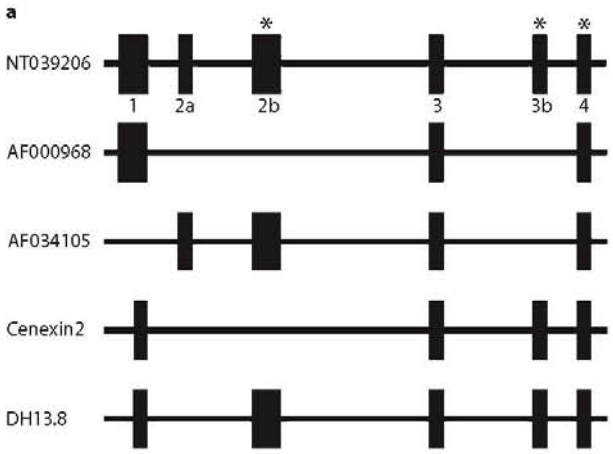

Fig. 1. (**a**) Schematic drawing of the exon-intron structure of the 5′ region of the *Odf2* gene (NT039206) and of alternative splice products. AF000968 and AF034105 were isolated from mouse testis and DH13.8 from NIH3T3 cells. Translational start codons are found in exons 2b, 3b and 4 and are marked by asterisks. (**b**) The N-terminal amino acid sequence alignment of these cDNA clones is shown. Protein accession numbers AAB65157.1, AAB87525.1, AAF80473.2, AAZ39869.1 correspond to nucleotide accession numbers AF000968, AF034105, AF162756 (Cenexin2) and DQ091769 (DH13.8), respectively. Amino acid sequences are derived from exon 2b (grey underlay), 3, 3b (grey underlay), 4, 5 (grey underlay), and 6 of the mouse gene.

from testis. Additionally, DH13.8 (AAZ39869.1) comprises amino acids encoded by exon 3b which formerly seems to be unique for Cenexin (hence this sequence of 36 amino acids is here referred as 'Cenexin insertion').

Divergent N-terminal regions of ODF2/Cenexin proteins

The nucleotide sequences of *Odf2* 5′ RACE products and of annotated *Cenexin* and *Odf2* cDNA clones were translated and N-terminal regions were aligned (Fig. 2). Generally, *Odf2* cDNA clones from testes libraries encode two different protein groups which differ by their most N-terminal sequences. Proteins AAB87525.1 (protein acc. no., corresponding to nucleotide accession no. AF034105) from mouse testis (Hoyer-Fender et al., 1998), AAB66337.1 (nucleotide accession no. AF012549) (Petersen et al., 1999) from human testis, AAC08408.1 (nucleotide acc. no. AF053971) (Schalles et al., 1998) from rat testis, and BAE00649.1 from *Macaca fascicularis* testis start with amino acid sequence MSASS corresponding to the translational start in exon 2b. Proteins AAB65157.1 (nucleotide acc. no. AF000968) (Hoyer-Fender et al., 1998) from mouse testis, AAC08409.1 (nucleotide acc. no. AF053970) (Petersen et al., 1999) from human testis, and AAC53134.1 (nucleotide acc. no. U62821) (Brohmann et al., 1997) from rat testis start with amino acids MKG corresponding to the translational start in exon 4. CAA64567.1 (nucleotide acc. no. X95272) from rat testis (Turner et al., 1997) seems to be an N-terminal truncated version as it starts with MRLS in exon 5. However, apart from amino acid alignment in Fig. 2, there is another group of proteins containing an additional sequence of 36 amino acids (here referred as 'Cenexin insertion') starting with MKDR. This group comprises AAF80473.2 (nucleotide acc. no. AF162756; Cenexin 2 from rat, Lange and Gull, 1995), AAH57001.1 (nucleotide acc. no.BC057001, Strausberg et al., 2002) from mouse brain, NP_0010128 (nucleotide acc. no. NM001012799) from chicken bursal lymphocytes (Caldwell et al., 2005), and ODF2 5′ RACE products from NIH3T3 cells DH8.5 (AAZ39870.1), DH9.5 (AAZ39867.1, nucleotide accession no.DQ091767), DH11.7 (AAZ39868.1, nucleotide accession no. DQ091768), and DH13.8 (AAZ39869.1). Cenexin 1 from rat (AAF80472.1/AF162755) seems to be derived from an incomplete cDNA clone as it is a more truncated version. 5′ RACE products DH9.5 and DH11.7 show a truncated exon 4 or a complete exon 4 skipping (DH11.7). With the exception of AAH10629 from human retinoblastoma that starts with MKG in exon 4, most ODF2/Cenexin proteins from somatic tissues/cells seem to contain additional N-terminal sequences which are derived from exon 3b, whereas in those from testicular tissues this sequence is mostly missing. There are only two reports concerning the presence of the 36 amino acid sequence starting with MKDR (or MKNR) in ODF2 derived from testis: AAQ73195 from human testis and AAH87396.1 from *Xenopus laevis* testis.

Fig. 2. Amino acid alignment of ODF2 derived from testis cDNA from mouse (AAB65157.1, AAB87525.1), human (AAC08409.1, AAB66337.1, AAQ73195.1), rat (CAA64567.1, AAC53134.1, AAC08408.1), *Macaca fascicularis* (BAE00649.1), and *Xenopus laevis* (AAH87396.1), and from cDNA from somatic tissues or cells, human eye (AAH10629.1), rat Cenexin 1 (AAF80472.1) and 2 (AAF80473.2), mouse brain (AAH57001.1), 5′ RACE ODF2 products from NIH3T3-cells AAZ39870.1 (DH8.5), AAZ39867.1 (DH9.5), AAZ39868.1 (DH11.7), AAZ39869.1 (DH13.8), and chicken bursal lymphocytes (NP_0010128).

To summarize, ODF2/Cenexin proteins from somatic versus testicular cells seem to differ in a short stretch of 36 amino acids at the N-terminal end ('Cenexin insertion'). Whereas the presence of these amino acids found first in Cenexin 2 seems to be characteristic for somatic ODF2/Cenexin proteins, they are missing in most testicular ODF2 proteins.

Expression of Odf2 isoforms in mouse tissues

We therefore asked whether the 'Cenexin insertion' is exclusively present in somatic ODF2 proteins suggesting possible functional differences. To check whether Odf2 isoforms show a specific expression pattern, we amplified the 5′ end from cDNA prepared from mouse tissues. First PCR reaction was performed with primer pair 13.8 EcoRI for and DH1rev (Fig. 3) resulting in amplification of those transcripts that contain exon 2b. As control the full length Odf2 clone 13.8NC (complete) was used resulting in a PCR product of 822 nt. In all somatic tissues but not in testis two PCR products of about equimolar amounts are found (Fig. 3a). One product corresponds to the PCR product amplified from 13.8NC (complete) which includes the nucleotide sequences of exon 3b encoding the 'Cenexin insertion' of 36 amino acids (aa). The size of the shorter PCR product matches the PCR product from testis cDNA of about 700 nt, that was expected when exon 3b is missing which was additionally confirmed by sequencing. However, a nested PCR on first PCR products using primer pair Insfor/DH1rev revealed one product of about 677 nt in all tissues including testis (Fig. 3b). Sequencing of PCR products from testis, ovary, kidney and brain confirmed the presence of exon 3b. The presence of a PCR product of about 677 nt in all tissues analysed supported the view that transcripts comprising exon 3b and hence the 'Cenexin insertion' are not restricted to specific tissues. However, in testis transcripts without exon 3b sequences (encoding the 'Cenexin insertion') are obviously predominant (Fig. 3a). Additionally, ODF2 proteins starting with the N-terminal amino acids MSASS derived from exon 2b are also not restricted to testis isoforms (Fig. 1b). Tissue specificity of spliced isoforms could therefore not be substantiated.

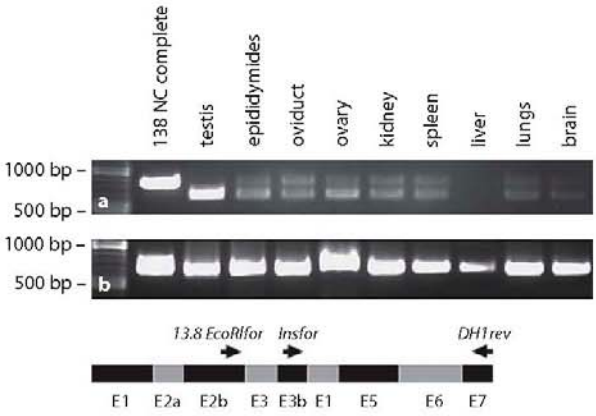

Fig. 3. RT-PCR on RNA isolated from mouse tissues demonstrates that transcripts comprising exon 2b are present in all tissues (**a**) as well as transcripts containing exon 3b (**b**). First PCR was performed with primer pair 13.8 EcoR1 for/DH1rev (**a**), and secondary PCR on first PCR products was performed with primer pair Insfor/DH1rev. Positions of primers are shown below.

Discussion

ODF2 has been located chromosomally to 9q34.11 in man (Shao et al., 1998), to 3q11→q12 in rat (Hoyer-Fender et al., 2003), and to mouse chromosome 2 band B (http://www.ncbi.nlm.nih.gov/mapview) indicating therefore that *ODF2* most likely is a single copy gene.

We have shown here that *Odf2* and *Cenexin* are splice variants which are derived most likely from the same gene. Additionally, we found a novel exon (exon 3b) not described before in *Odf2* gene sequence (Hoyer-Fender et al., 2003). In exon 3b a further translational start is present resulting in a sequence of 36 aa starting with MKDR (referred here as 'Cenexin insertion'). With a few exceptions most ODF2 isoforms from testes start with MSASS derived from exon 2b but do not contain the 36 aa 'Cenexin insertion', or are more truncated proteins that start with MKG from exon 4. On the contrary, somatic ODF2 isoforms mostly seem to contain the 36 aa insertion found first in Cenexin (Lange and Gull, 1995). Exceptions apply to sequences derived from human retinoblastoma (AAH10629.1) which starts with MKG (from exon 4) and to two testis derived sequences, AAQ73195.1 from human and AAH87396.1 from *Xenopus*, that start with the 36 aa 'Cenexin insertion' (from exon 3b). One possible explanation might be that the retinoblastoma derived sequence has an incomplete 5′ end. However, RT-PCR on RNA isolated from a variety of mouse tissues revealed that transcripts comprising exon 2b are present in all tissues analysed. In somatic tissues transcripts containing the 'Cenexin insertion' of 108 nt (derived from exon 3b) are found at about equimolar amounts to transcripts without this insertion. In testis, the predominant transcripts are those comprising exon 2b but without exon 3b. Our results support the view that transcripts comprising exon 3b (encoding the 'Cenexin insertion') are not restricted to specific tissues. However, taking into consideration annotated ODF2 sequences (Fig. 2), ODF2 proteins starting with the 'Cenexin insertion' seem to be predominant in somatic tissues whereas in testis most ODF2 sequences do not contain the 'Cenexin insertion'. Testis tissue consists of a huge amount of germ cells aside from a rather few somatic cells (e.g. Sertoli cells and Leydig cells among others). The different abundance of spliced isoforms in testis therefore could just reflect the amount of germ cells versus somatic cells, i.e. predominant *Odf2* transcripts in testis might be derived from germ cells and specifically from those that undergo sperm tail formation, whereas minor transcripts, i.e. transcripts comprising the 'Cenexin insertion' are derived from somatic cells. This would additionally account for the two exceptional *Odf2* sequences comprising the 'Cenexin insertion' found in human and *Xenopus laevis* testis.

The 'Cenexin insertion' of 36 aa (starting with MKDR) contains three predicted phosphorylation sites (one Ser, two Thr) which fall into a forkhead domain interaction motif 1 and a class IV WW domains interaction motif (http://www.expasy.org). WW domain-containing proteins are involved in many cellular processes, e.g. mitotic regulation. The forkhead-associated domain is involved in signal transduction, cell cycle control and DNA repair. However, whether the additional 36 aa sequence of ODF2/Cenexin proteins is a protein-protein interaction domain has to be elucidated in future research.

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We thank Robert Fink for excellent technical assistance and Elsbeth Blabusch for secretarial help. Sequences have been submitted to GenBank.

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3.2. Molecular dissection of ODF2 / Cenexin revealed a short stretch of amino acids necessary for targeting to the centrosome and the primary cilium

In this part, the cellular localizations and the functional differences of the isolated splice variants were studied. The function of the N-terminal stretch in context to the cellular localization of the protein is described. Furthermore, the testicular expression of Odf2 splice variants was investigated.

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Author contributions to the practical work:

Daniela Hüber: Cloning of 13.8 and the "insertion" alone into the eukaryotic expression vector pEGFP-N1 (clontech), comparative immunofluorescence microscopy of all expression constructs to study cellular localization and further immunofluorescence work to determine protein associations to confine functional dispositions.
Stephanie Geisler: RT-PCR analysis on mouse testes
Sebastian Monecke: Cloning of the full - length 13.8NC construct in pEGFP-N1 (Clontech) and determination of its cellular localization by immunofluorescence microscopy (partial).

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Molecular dissection of ODF2/Cenexin revealed a short stretch of amino acids necessary for targeting to the centrosome and the primary cilium

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Abstract

The outer dense fiber protein ODF2 is the major component of the sperm tail cytoskeleton and a critical component of the mature centriole of the centrosome. Centriole maturation involves the formation of appendages and the recruitment of ODF2/Cenexin. ODF2 and Cenexin are alternative splice variants that differ in a short stretch of amino acids at their N-terminal regions encoded by exon 3b. Whereas *Cenexin* is ubiquitously expressed, *Odf2* is the predominant transcript of testes [Hüber, D., Hoyer-Fender, S., 2007. Alternative splicing of exon 3b gives rise to ODF2 and Cenexin. Cytogenet. Genome Res. 119, doi:10.1159/000109621]. Here, we show that testicular expression of *Odf2* correlates with spermiogenesis and ongoing sperm tail formation thus implicating functional differences between ODF2 and Cenexin. By generation of a series of ODF2/Cenexin deletion constructs fused to GFP and inspection of their subcellular localization in transfected NIH3T3 cells we found that a peptide of 42 amino acids specific for Cenexin is necessary for targeting ODF2/Cenexin to the centrosome and the primary cilium. Additionally, this region is also necessary for the formation of ODF2/Cenexin fibers that are associated with acetylated microtubules. Centrosomal targeting of ODF2/Cenexin does not depend on dynein-mediated transport further supporting an alternative targeting mechanism. However, part of the C-terminal coiled-coil region of ODF2 is also important in centrosomal/ciliary targeting and fiber formation presumably by supporting self-association and the formation of higher-order structures.

Keywords: Primary cilia; Centrosome; Sperm tail; Outer dense fibers; Acetylated tubulin

Introduction

The cytoskeleton of the mammalian sperm tail is characterized by nine to seven accessory fibers that accompany the axonemal tubuli doublets on their outer site. These outer dense fibers (ODFs) are composed of more than a dozen different proteins (Vera et al., 1984; Oko, 1988; Petersen et al., 1999) of which only a few have been identified including ODF2 as its major component. The ODFs seem not to be involved in active motility but are important for the stability and the elastic recoil of the sperm tail as well as for support of the flagellar beat (Baltz et al., 1990; Lindemann, 1996). Impaired development of the ODFs has been described as a major cause of tail abnormalities in infertile men (Haidl et al., 1991) indicating an important function in

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sperm motility and/or morphology. *Odf2* has first been described as testicular transcript with abundant expression in postmeiotic male germ cells (Petersen et al., 1999; Brohmann et al., 1997; Shao et al., 1997; Turner et al., 1997; Hoyer-Fender et al., 1998; Schalles et al., 1998). However, expression analyses by RT-PCR and EST-Blast revealed transcription of *Odf2* in a lot of somatic tissues including heart and brain, as well as in carcinoma cell lines (Hoyer-Fender et al., 2003). In support of these findings, ODF2 has been identified as an intrinsic component of the centrosome in animal species as well as being crucial for the generation of primary cilia (Nakagawa et al., 2001; Hoyer-Fender et al., 2003; Ishikawa et al., 2005).

The centrosome is located at the vicinity of the nucleus in the cell center. It is the major microtubule-organizing center (MTOC) of the eukaryotic cell and functions to generate the mitotic spindle during mitosis. Moreover, the centrosome seems to play an important role in orchestrating cell cycle progression (Kellogg et al., 1994; Stearns and Winey, 1997; Zimmermann et al., 1999; Bornens, 2002; Fry and Hames, 2004). In animal cells, the centrosome is composed of a pair of centrioles surrounded by an electron-dense cloud of pericentriolar material, the pericentriolar matrix (PCM) (Kellogg et al., 1994; Stearns and Winey, 1997; Zimmermann et al., 1999; Bornens, 2002). Although centrioles are mainly built from α/β-tubulin dimers, each centrosome contains two unequal centrioles of which the older or mother centriole is characterized by the presence of distal and subdistal appendages. Among those proteins that specifically associate with the appendages of the mother centriole are ODF2 (Nakagawa et al., 2001) and Cenexin (Lange and Gull, 1995). ODF2-deficient mother centrioles lack their appendages and are unable to generate primary cilia (Ishikawa et al., 2005) pointing to the significance of ODF2 in cilia formation.

Primary cilia, i.e. eukaryotic cilia and flagella in general, are anchored to the cell by the basal body, which develops from the mother centriole of the centrosome (Wheatley et al., 1996). They project from the surface of cells consistent with their recent identification as essential sensory organelles (Huangfu et al., 2003; Pazour and Witman, 2003; Corbit et al., 2005; Haycraft et al., 2005; Huangfu and Anderson, 2005; May et al., 2005; Pan et al., 2005; Schneider et al., 2005; Michaud and Yoder, 2006). The presence of a primary cilium is associated with the establishment of polarity and differentiation of the cell. Entry into the cell cycle is in many cells preceded by ciliary resorption whereas exit from mitosis is accompanied by ciliary assembly (Tucker et al., 1979; Rieder et al., 1979). Disassembly of the cilium and the basal body thus frees the centrioles to function as the organizing center of the mitotic spindle. Primary cilia are found on most epithelial and stromal cells throughout the mammalian body (for a comprehensive list of cells and tissues containing cilia see http://members.global2000.net/bowser/cilialist.html). They are immotile with a $9+0$ microtubule arrangement (nine peripheral doublet microtubules without the two central singlet microtubules found in motile cilia) and are found singly on cells in contrast to motile $9+2$ cilia which usually occur in groups. The observation that certain proteins associated with cilia-related diseases are localized to the primary cilium as well as to the basal body and to the centrosome further strengthens the view that all three organelles are structurally and functionally united (Pan et al., 2005; Pazour and Rosenbaum, 2002; Davenport and Yoder, 2005; Badano et al., 2006).

ODF2 and Cenexin are marker proteins for the mature centriole of the centrosome that eventually gives rise to the basal body of the primary cilium. Both proteins differ by a short stretch of amino acids at the N-terminal region which is present in Cenexin and absent in ODF2. This Cenexin insertion is encoded by exon 3b and starts with codons for the amino acids MKDR. Alternative splicing therefore gives rise to ODF2 or Cenexin. Whereas Cenexin showed a constant expression in all tissues, *Odf2* was upregulated in testes (Hüber and Hoyer-Fender, 2007). Moreover, we show here, that upregulation of *Odf2* expression correlated with spermiogenesis and ongoing sperm tail formation suggesting functional differences between ODF2 and Cenexin proteins. We therefore asked whether ODF2/Cenexin proteins could be dissected into functional domains. We generated a series of deletion constructs that were fused in-frame to GFP. Expression of these fusion constructs in NIH3T3 cells revealed a remarkable distinct subcellular localization pattern. We demonstrate that the Cenexin insertion, a peptide of 42 amino acids at the N-terminal region of Cenexin and of the ODF2/Cenexin isoform 13.8 isolated from NIH3T3 cells (Hüber and Hoyer-Fender, 2007), is necessary for targeting ODF2/Cenexin isoforms to the centrosome and the primary cilium. Additionally, this region is also necessary for ODF2/Cenexin fiber formation and the association with acetylated microtubules, which are found in centrosomes, primary cilia, and axonemata. However, centrosomal/ciliary targeting and intracellular fiber formation depends on the C-terminal region of ODF2 as well since the Cenexin insertion by itself is unable to mediate correct targeting and fiber formation. As the C-terminal region of ODF2 forms a predicted coiled-coil structure its contribution most probably relies on promoting self-association and the formation of higher-order structures. Targeting of ODF2/Cenexin to the centrosome does not depend on dynactin/dynein-mediated minus-end-directed transport to the centrosome supporting additionally the view of an alternative mechanism, which very likely could rely on the binding to acetylated microtubules.

Materials and methods

RT-PCR

Total RNA from mouse testes was prepared using peqGOLD TriFast reagent and digested with RQ1 DNase. cDNA was generated using oligodT primer and AMV reverse transcriptase. Amplification of the *Odf2* 5′ region was performed with primer pair 13.8-EcoRI-for (GGAATTCATGTCCGCCTCATCCTCAGGC) and DH1-rev (CAGCTTCCCGATGGTATCCTTCAAG). A nested PCR was performed on first PCR products with primers Ins-for (CTTCAACTCCCCCCTTACATG) and DH1-rev.

The following primers were used for RT-PCR reactions to amplify *Scp3* (synaptonemal complex protein 3): GACGGTACCATGCTTCGAGGGTGTGGG/GACGGATCCAATAACATGGATTGAAG, *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase): CACCACCAACTGCTTAGCC/CGGATACATTGGGGGTAGG, and *Odf1* (outer dense fiber protein 1): GAGCTCAAGCTTTGGCCGCACTGAGTTGTC/CCGCGGTACCCAAGATCATCTTCCTACA. PCR products were gel purified and sequenced (MWG Biotech, Germany).

Subcloning and transfection

The N-terminal region of RACE clone DH13.8 was amplified by PCR using primer pair 13.8-EcoRI-for and 13.8-Sma-rev (ATCCCGGGCGGAATCCTCTTTGCAGTG) and cloned in-frame into pEGFP-N1 (Clontech, Palo Alto) to generate clone 13.8. The N-terminal region of 13.8 was cloned in-frame to the C-terminal part of *Odf2NC* in pEGFP-N1 (Donkor et al., 2004) by double digestion with EcoRI and MscI generating 13.8NC (compl.). The following subclones (see Fig. 2) were generated by PCR out of clone 13.8NC (compl.): N-terminal using primer pair 13.8-EcoRI-for and Ins-Sma-rev (TCCCGGGCCTGGCTGCTGGTCGCC), Cenexin insertion using primer pair Ins-Eco-for (GGAATTCATGAAGGACCGCTCTTC) and Ins-Sma-rev, and Cenexin using primer pair Ins-Eco-for and Odf2C (TGGATCCCGGGTCGGTAAGCGGGCCCCTGC). The human cDNA clone (AF012549) (Petersen et al., 1999) was amplified with primer pair 13.8-EcoRI-for and Odf2C. All fragments were cloned in-frame into pEGFP-N1 and the correct reading frame was verified by sequencing. *Odf2* constructs were transfected into NIH3T3 cells using Transfectin (BioRad, Munich, Germany).

Immunofluorescence microscopy

NIH3T3 cells were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum and antibiotics at 37 °C and 5% CO_2. Transfected cells were grown on coverslips, fixed alternatively in methanol or in 3.7% formaldehyde (freshly prepared from paraformaldehyde) in phosphate-buffered saline (PBS) at pH 7.5. Cells were preincubated in 0.2%Triton X-100 in PBS for 5 min, 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 antibodies was done at 37 °C for 1 h. Monoclonal anti-γ-tubulin (GTU88), anti-acetylated-tubulin (6-11B-1), and anti-mouse Cy3 antibodies were supplied from Sigma Biosciences (St. Louis, MO). Anti-human Golgin-97 and anti-mouse Alexa-488 were from Molecular Probes (Eugene, OR). Anti-rabbit Cy3 antibody was from Amersham. Human SIRT2 (in pcDNA-DEST-40) (Michishita et al., 2005) was detected with anti-V5 antibody (ab9116; Abcam, Cambridge, UK), human HDAC6 (Grozinger et al., 1999) with anti-FLAG-FITC (F4049, Sigma), and human dynamitin (pCMVH50) (Echeverri et al., 1996) with anti-myc antibody (9E10). DNA was counterstained with DAPI. Images were taken by confocal microscopy (LSM 510, Zeiss), and processed using Adobe Photoshop 5.0.

Results

Odf2 transcripts not comprising exon 3b accumulate with ongoing spermiogenesis and sperm tail formation

In order to find out whether expression of *Odf2* corresponds to spermatogenic progression and, more specifically, to sperm tail formation, we took advantage of the characteristic progression of spermatogenic differentiation in the postnatal mouse testis resulting in lack or enrichment, respectively, of particular germ cell types at specific times after birth (Nebel et al., 1961). Meiosis starts around day 10, early round spermatids are present approximately 10 days later, and the early elongating spermatid stage is reached at about day 26. Spermatogenic progression moreover was monitored by RT-PCR of marker genes. Expression of *Scp3* was used to monitor meiosis, and expression of *Odf1* to detect post-meiotic stages and progression of sperm tail formation (Burmester and Hoyer-Fender, 1996). *GAPDH* was used as a general marker and demonstrated that equal amounts of cDNA were used for RT-PCR (Fig. 1b). RT-PCR with primer pair 13.8-EcoRI-for/DH1-rev verified *Odf2* transcripts at 20 and 25 days post partum (dpp) and confirmed predominance of alternatively spliced *Odf2* transcript missing exon 3b in testis. In the control reaction (c), 13.8NC (compl.) was used as template resulting therefore in a PCR product including exon 3b which is all in all somewhat larger than the testis PCR product (Fig. 1b) (Hüber and Hoyer-Fender, 2007). However, secondary PCR reaction on first PCR products revealed

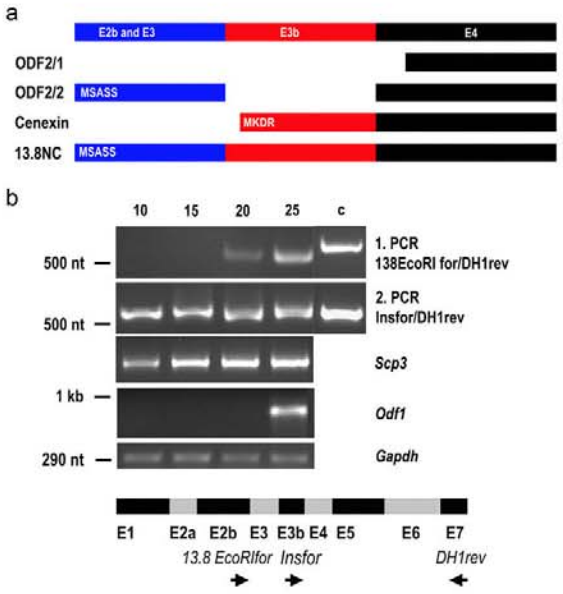

Fig. 1. (a) Schematic drawing of the N-terminal amino acid sequences of ODF2/1 and ODF2/2, Cenexin, and ODF2/Cenexin isoform 13.8 NC. ODF2/1 and ODF2/2 were isolated from testis (Brohmann et al., 1997). The color code corresponds to that in Fig. 2. (b) Expression of the *Odf2* splice variant missing exon 3b sequences (1. PCR) is upregulated with ongoing spermiogenesis and sperm tail formation, whereas transcripts comprising exon 3b sequences (2. PCR) are present throughout testis development. Control (c): PCR on the full-length template. *Scp3* and *Odf1* were used as meiosis and sperm tail formation markers, respectively. *GAPDH* served as loading control. Localization of PCR primer pairs in exon1 to 7 (E1–E7) of *Odf2/Cenexin* is shown below.

that transcripts comprising exon 3b are nonetheless present throughout testis development (Fig. 1b, 2. PCR with primer pair Ins-for/DH1-rev). Additionally, these transcripts did not vary within testis development but instead seemed to be synthesized throughout at about equimolar amounts. In contrast, the predominant testicular transcript (without exon 3b) accumulated concurrently with ongoing spermiogenesis and sperm tail formation (compare Fig. 1b, 1. PCR and the expression of *Odf1*). Sequencing of PCR products confirmed the absence of exon 3b in predominant testicular transcript but the presence of exon 3b in the ubiquitously expressed transcript.

The Cenexin insertion is necessary but not sufficient for centrosomal targeting

We next asked whether the additional amino acids encoded by exon 3b and located at the most N-terminal end of Cenexin (see Fig. 2, amino acids accentuated in red) might have any influence on the function or location of the protein. We therefore fused different parts of ODF2/Cenexin to GFP and transfected the constructs into NIH3T3 mouse cells. Excitingly, the full-length construct starting with MSASS at the N-terminus followed by the Cenexin insertion (13.8NC (compl.)) showed a very specific location to centrosomes. However, centrosomal location seemed not to be restricted to the mature centriole (Fig. 2a c) as the full-length construct was also recruited to the basal body and the primary cilium (Fig. 2d f). In order to narrow down the protein domain responsible for centrosomal location, we made a series of GFP fusion constructs (Fig. 2). Human testis ODF2 (AAB66337.1) which starts with amino acid sequence MSASS but does not contain the Cenexin insertion showed a more or less uniform distribution (Fig. 2g i). The N-terminal regions themselves, without the C-terminal coiled-coil region of ODF2, did also not locate to the centrosome (Fig. 2p u) but in contrast showed a very similar and even, cytoplasmic and nuclear distribution. There was no concentration at the centrosomes as revealed by anti-γ-tubulin staining (Fig. 2q, t). The N-terminal 322 amino acids of ODF2/Cenexin comprising the N-terminal end of 13.8NC (compl.) followed by 232 aa of the coiled-coil region (yielding construct 13.8) was found in the nucleus and with a fine granular staining inside the cytoplasm (Fig. 2 inset in o). Though 13.8 located to the centrosomes (Fig. 2m o) its fluorescence intensity and hence its amount at the centrosome was much less compared to the full-length construct (13.8NC (compl.)). Cenexin, consisting of the entire C-terminal coiled-coil region of ODF2 and the N-terminal Cenexin insertion, however, was restricted to the centrosome (Fig. 2j l), which was verified by anti-γ-tubulin staining (Fig. 2k, l). These results show that the Cenexin insertion is necessary for centrosomal targeting. However, as the Cenexin insertion by itself did not locate to the centrosome a great part of the C-terminal coiled-coil region of ODF2 seemed to be important as well for centrosomal targeting.

Association of ODF2/Cenexin proteins with acetylated microtubules

In the majority of cells, the full-length construct 13.8NC (compl.) located exclusively to the centrosomes and, if present, to the primary cilium and its basal body (Fig. 3a c). However, a variable portion of cells showed a striking granular or fibrillar pattern (Fig. 3d i). Anti-α-tubulin staining revealed colocalization of 13.8NC (compl.) to a subset of microtubules (Fig. 3g i). We therefore asked whether these microtubules are modified. Staining for acetylated tubulin revealed that 13.8NC (compl.) indeed tagged the primary cilium

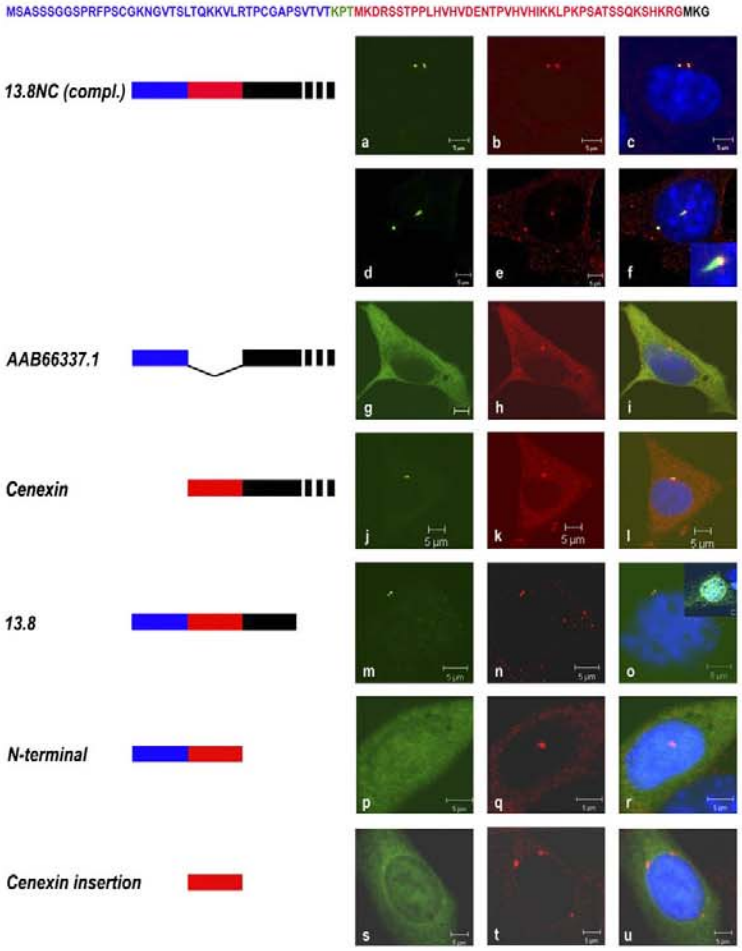

Fig. 2. Localization of ODF2/Cenexin-GFP fusion proteins in NIH3T3 cells. 13.8NC (compl.) comprising the entire N-terminal region was directed to the centrosome and to the primary cilium (a–f). Inset in (f) shows an enlargement of the primary cilium. The complete N-terminal region and part of the C-terminal region yielding a protein of 322 amino acids (construct 13.8; m–o) is also found in the centrosome albeit with a much weaker intensity. Moreover, 13.8 also located to the nucleus and showed a fine granular staining pattern inside the cytoplasm (inset in o). Cenexin, comprising the Cenexin insertion and the coiled-coil region of ODF2, however, located to the centrosome (j–l). All other constructs missing either the Cenexin insertion (g–i) or the C-terminal coiled-coil region of ODF2 (p–u), exhibited a rather even distribution with no enrichment at the centrosome. The amino acid sequence of the N-terminal region shown corresponds to sequence AAZ39869.1. Amino acids KPT (in green) are present exclusively in those proteins that contain both the blue (N-terminal end of splice variant ODF2/2) and the red (Cenexin insertion) parts. Amino acids KSHKRG at the C-terminal end of the Cenexin insertion are also found in those proteins starting with MSASS but missing the Cenexin insertion. The broken black bar represents the full-length C-terminal region of ODF2 derived from testis. AAB66337.1 is human ODF2. Red: γ-tubulin staining (besides n, o which is acetylated tubulin), green: GFP fluorescence, blue: DAPI counterstaining. Bars: 5 µm.

(Fig. 3a c). Moreover, the fibrillar pattern of the GFP-fusion protein followed approximately the pattern of acetylated microtubules (Fig. 3d f). In the absence of distinct acetylated microtubules inside the cytoplasm the GFP fusion to 13.8NC (compl.) sometimes revealed a granular staining pattern (not shown). The C-terminally truncated fusion protein 13.8-GFP likewise showed a granular or fibrillar staining pattern (although much smaller sized) (Fig. 2 inset in o; Fig. 3j l). These fibers are also roughly associated with acetylated microtubules (Fig. 3j l). We therefore asked whether tubulin deacetylation influences the location of the full-length GFP fusion protein 13.8NC (compl.). Tubulin deacetylation was performed in vivo by overexpression of hSIRT2 and/or hHDAC6 and checked by antibody staining. The human sirtuin SIRT2 (hSIRT2) is a

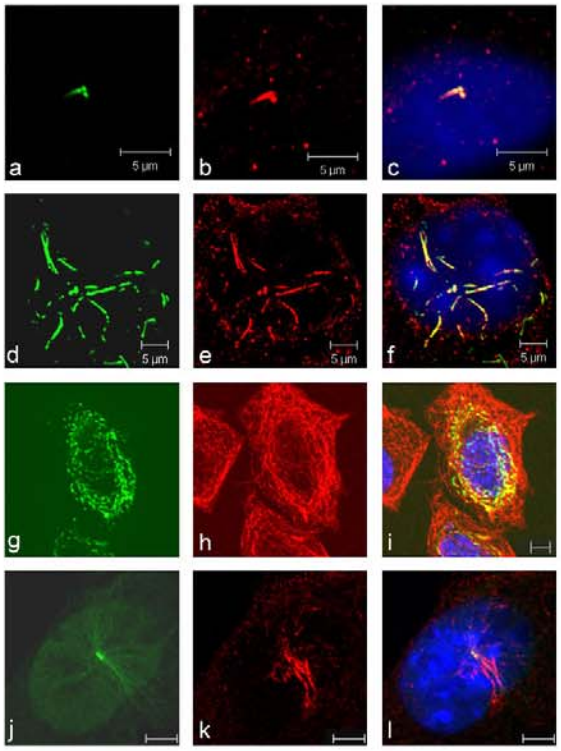

Fig. 3. Colocalization of GFP fusion proteins 13.8NC (compl.) (a–f) and 13.8 (j–l) with acetylated tubulin. (a–c) 13.8NC (compl.) localized to the centrosome/basal body and the primary cilium. Colocalization of 13.8NC (compl.) with a subset of microtubules was verified by detection of α-tubulin (g–i). Constructs were transfected into NIH3T3 cells, and fixed cells were incubated with anti-acetylated-tubulin antibody (a–f and j–l) or with anti-α-tubulin antibodies (g–i). Antibodies were detected with Cy3-labeled secondary antibody (red), and nuclei were stained with DAPI (blue). GFP fluorescence in green. Bars: 5 µm.

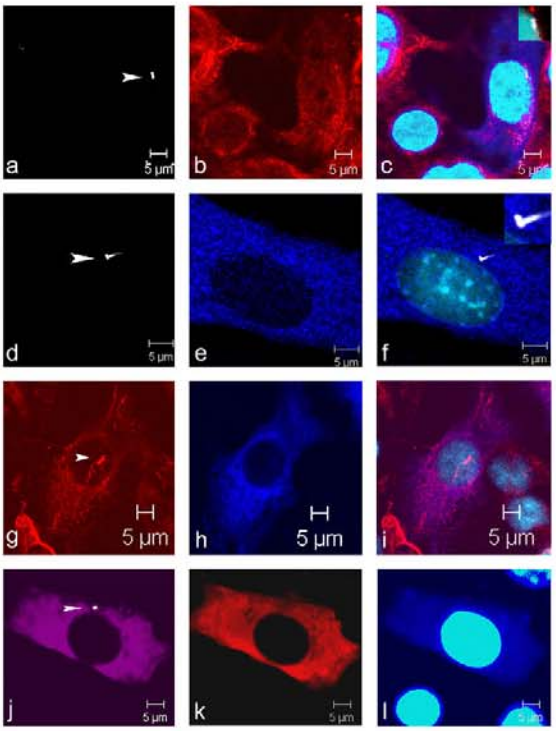

Fig. 4. Tubulin deacetylation by overexpression of SIRT2 and/or HDAC6 impaired neither the centrosomal (a–c and j–l) nor the ciliary (d–f) localization of the 13.8NC (compl.)-GFP fusion protein. Overexpression of SIRT2 correlated with deacetylated cytoplasmic microtubules (a–c and g–l) whereas the primary cilium remained highly acetylated (Fig. 4g–i). Centrosomal recruitment of 13.8NC (compl.)-GFP is also not impaired by overexpression of both deacetylases SIRT2 and HDAC6 in the same cell (j–l). Nuclear counterstaining in light blue, GFP fluorescence in white, antibody staining against acetylated tubulin in red, SIRT2 overexpression in dark blue, HDAC6 overexpression in pink (j). Insets show enlargements of centrosomes (c) and the primary cilium (f). Arrowheads: centrosomes (a, j), basal body/primary cilium (d), and primary cilium (g). Merge images c,f,i.

tubulin deacetylase that specifically deacetylates α-tubulin on lysine-40 but does not change the microtubule network (North et al., 2003). hSIRT2 is predominantly cytoplasmic (Fig. 4) consistent with previous reports (Michishita et al., 2005; North et al., 2003). Overexpression of hSIRT2 reduced the acetylation state of cytoplasmic microtubules (Fig. 4b, c and g, h) although the primary cilium and the centrosome remained highly acetylated (Fig. 4g i). Overexpression of hSIRT2 impaired neither the centrosomal nor the ciliary localization of the GFP fusion 13.8NC (compl.) (Fig. 4a c and d f, respectively), while cytoplasmic microtubules are no longer acetylated. However, tubulin deacetylation by overexpression of deacetylases impaired fiber formation of ODF2 isoform 13.8NC (compl.). HDAC6 has been described as a class II histone deacetylase that deacetylates α-tubulin in vitro and in vivo (Matsuyama et al., 2002; Zhang et al., 2003). Concurrent expression of both deacetylases, SIRT2 and HDAC6, did not hamper the centrosomal localization of 13.8NC-GFP albeit cytoplasmic microtubules were no longer acetylated (Fig. 4j l).

Centrosomal targeting of isoform 13.8NC (compl.) does not depend on dynactin-mediated minus-end transport

ODF2 has been described as a centrosomal scaffold protein, the centrosomal localization of which is not affected by nocodazole-driven microtubule depolymer-

ization (Nakagawa et al., 2001). Accordingly, ODF2 is localized at centrosomes in a microtubule-independent manner. However, although disruption of microtubules clearly demonstrated that they are unimportant for ODF2 to stay at the centrosome, it is an inappropriate experiment concerning the recruitment of ODF2 to the centrosome. We asked whether recruitment of ODF2 isoform 13.8NC (compl.) to the centrosome depends on the microtubular minus-end-directed transport driven by dynein. We took advantage of the functional impairment of dynein by overexpression of dynamitin. Dynein and dynactin are key players in microtubule organization, centrosome integrity, and centrosome assembly. Cytoplasmic dynein is a minus-end directed microtubule motor that works in conjunction with dynactin. Dynactin is a multiprotein complex, which appears to serve as an accessory factor in cytoplasmic dynein function. Overexpression of dynamitin, a subunit of the dynactin multiprotein complex, impairs both dynactin and dynein function in the cell (Echeverri et al., 1996; Burkhardt et al., 1997) resulting amongst others in perturbed centrosome integrity (Quintyne et al., 1999). Whereas the microtubular cytoskeleton was not affected by dynamitin overexpression the Golgi distribution was disrupted (Burkhardt et al., 1997). By overexpression of dynamitin in NIH3T3 cells we found intact microtubules (Fig. 5a c) but a dispersed Golgi (Fig. 5d f) just as described by Burkhardt et al. (1997). However, overexpression of dynamitin did not interfere with centrosomal location of ODF2/Cenexin isoform 13.8NC (compl.) (Fig. 5g i). Recruitment of ODF2/Cenexin to the centrosome therefore most likely is not based on dynein-mediated minus-end-directed transport.

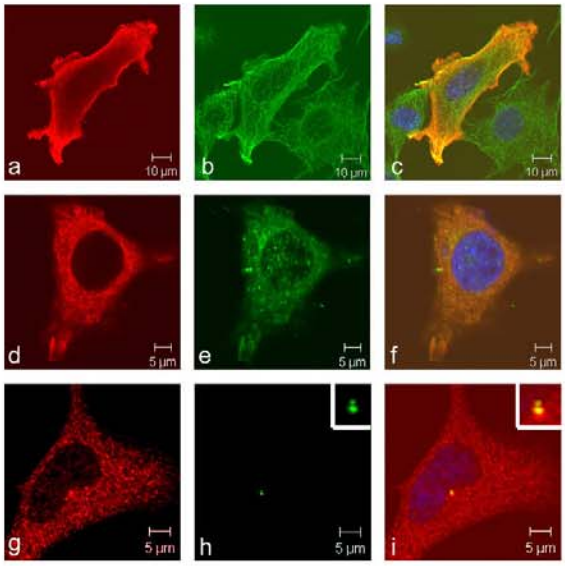

Fig. 5. Centrosomal recruitment of ODF2/Cenexin is not based on dynein-mediated transport. Overexpression of the dynamitin subunit did not prevent targeting of 13.8NC (compl.)-GFP to the centrosome (g–i). Overexpression of dynamitin did not affect the microtubular cytoskeleton (a–c) but disrupted the Golgi (d–f). Dynamitin expression in red (a, d, g), anti-α-tubulin (b), anti-Golgin-97 (e) and 13.8NC (compl.)-GFP (h) in green. Merge images including DAPI counterstain (c, f, i). Inset in (i): enlargement of the centrosome.

Discussion

Alternative splicing of exon 3b gives rise to ODF2 or Cenexin (Hüber and Hoyer-Fender, 2007). Exon 3b includes a further translational start resulting in a sequence of 36 aa starting with MKDR which is exclusively present in Cenexin (herein referred to as Cenexin insertion). ODF2 proteins start with MSASS derived from exon 2b, or are more truncated proteins that start with MKG from exon 4. ODF2 isoforms first isolated from testes do not contain the 36 aa Cenexin insertion. Whereas Cenexin has been found to be ubiquitously and equally expressed in all tissues analyzed, *Odf2* expression is upregulated in testes and correlates with ongoing spermiogenesis and sperm tail formation. This prompted us to look for putative functional domains in ODF2/Cenexin proteins. We therefore made a series of deletion constructs, fused them to GFP and investigated their expression pattern in NIH3T3 cells.

Transfection of *Odf2/Cenexin-GFP* fusion constructs into NIH3T3 mouse cells revealed that there are only three constructs being targeted to the centrosome. These are 13.8NC (compl.) and its C-terminal truncated version 13.8 as well as Cenexin. 13.8NC (compl.) and Cenexin both contain the entire C-terminal coiled-coil region of 591 aa but differ in their most N-terminal sequences. In contrast, the truncated protein 13.8 is identical to 13.8NC (compl.) in its N-terminal region but then comprises just the N-terminal 232 aa of the coiled-coil region. The coiled-coil region of ODF2 of 591 aa has been investigated in detail elsewhere (Donkor et al., 2004). Constructs comprising only this C-terminal coiled-coil part showed a cytoplasmic and/or nuclear localization with a more or less even distribution. Neither preferential nor exclusive centrosomal localization could be found. Though the coiled-coil region of 591 aa comprises the main part of the protein, the N-terminal amino acid sequence of 42 aa, namely the Cenexin insertion of 36 aa and the 6-aa sequence KSHKRG that links the Cenexin insertion to MKG (Fig. 2), is necessary for centrosomal and ciliary targeting. However, the N-terminal end on its own did not target to the centrosome and the primary cilium but instead requires at least part of the coiled-coil region. Moreover, it was not possible to narrow down a centrosomal targeting sequence as no shorter sequence could be identified that was sufficient for targeting the centrosome.

AKAP450 and Pericentrin share the PACT domain, a conserved region of about 90 aa at the C-terminal end that is a centrosomal targeting motif (Gillingham and Munro, 2000). The PACT domain anyway was not found in ODF2/Cenexin. Moreover, ODF2/Cenexin obviously seems not to share any other sequence motifs with known centrosomal/ciliary proteins. Flagellar/ciliary targeting domains had been reported previously mostly in species of the protozoans *Leishmania* and *Trypanosoma* (Godsel and Engman, 1999; Ersfeld and Gull, 2001; Nasser and Landfear, 2004; Tull et al., 2004). A ciliary membrane protein in higher eukaryotes is polycystin-2 which uses an RVxP motif for ciliary trafficking (Geng et al., 2005). The same motif has also been implicated in ciliary targeting of the cyclic nucleotide-gated channel subunit CNGB1b (Jenkins et al., 2006). An RVxP motif is not found in the N-terminal 86 aa region of ODF2/Cenexin isoform 13.8NC (compl.). However, ODF2/Cenexin proteins are associated with microtubules (Donkor et al., 2004) instead of membranes. The centrosomal/ciliary targeting region of ODF2/Cenexin therefore represents a domain that most probably is involved in targeting to the axonemal structures of eukaryotes.

The Cenexin insertion of 36 aa (starting with MKDR) contains three predicted phosphorylation sites (one Ser, two Thr) which fall into a forkhead domain interaction motif 1 and a class IV WW domains interaction motif (http://www.expasy.org). WW domain-containing proteins are involved in many cellular processes, e.g. mitotic regulation. The forkhead-associated domain is involved in signal transduction, cell cycle control and DNA repair. However, whether this additional 36-aa sequence of ODF2/Cenexin proteins is a protein protein interaction domain has to be elucidated in future research.

ODF2 proteins are predicted coiled-coil proteins with an almost overall coiled-coil structure (Lupas et al., 1991). However, regarding the largest protein encoded by 13.8NC (compl.), about 150 aa from the most N-terminal end do not have a coiled-coil structure. The coiled-coil region of ODF2 functions in self-association and in microtubule binding but does not interact directly with tubulin (Donkor et al., 2004). We have shown here that proteins containing an elongated N-terminal region, namely 13.8NC (compl.) and 13.8, associate with acetylated microtubular structures. Acetylated tubulin is found in the centrosomes, the primary cilium, and in a subset of cells inside the cytoplasm. Acetylation of α-tubulin is mostly associated with stable microtubular structures such as axonemata, and it occurs after microtubule assembly (Westermann and Weber, 2003). Our results demonstrate that association of ODF2/Cenexin with acetylated microtubules strictly depends on these additional N-terminal amino acids, namely the Cenexin insertion and the furthest N-terminal sequences starting with MSASS. However, self-association and the formation of fibers most likely reside in the C-terminal coiled-coil region. We therefore suspect that the N-terminal region associates directly or indirectly with acetylated microtubules, followed by the formation of higher-order structures mediated through the C-terminal coiled-coil region, thus causing aggregation at regions containing acetylated microtubules, e.g. centrosomes, primary cilia and axonemata. Omitting parts of the N-terminal region, and to be more specifically the Cenexin insertion, thus would prevent targeting to acetylated microtubules (as was shown for the human ODF2-GFP fusion construct AAB66337.1), omitting the C-terminal coiled-coil region totally prevents self-association and the formation of higher-order structures (as was shown for the GFP-fusions to the N-terminus and the Cenexin insertion), whereas omitting parts of the coiled-coil region does not prevent self-association but reduces it, resulting in very fine fibrillar structures (as shown for construct 13.8-GFP). The N-terminal sequence, and notably the Cenexin insertion, therefore most likely is important for targeting acetylated microtubules instead of being a centrosomal targeting motif per se. Moreover, centrosomal recruitment of ODF2/Cenexin does not depend on dynein/dynactin-mediated transport further supporting an alternative mechanism.

The primary cilium has taken center stage in a broad class of human diseases with pleiotropic manifestations that include autosomal dominant and recessive polycystic kidney disease, nephronophthisis, and Bardet-Biedl syndrome (Pan et al., 2005; Pazour, 2004). The proteins encoded by genes mutated in these diseases localize to the cilium complex. It has been shown that the ability to generate primary cilia depends on ODF2 (Ishikawa et al., 2005) implying that it is directly involved in cilia formation. However, its actual function, e.g. whether ODF2/Cenexin affects the formation or the stabilization of cilia is unknown.

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3.3. Functional characterization of splice variant 13.8NC: Interaction of Odf2 / Cenexin with the Retinoblastoma Protein (Rb)

In this part of my thesis I describe the interaction between Odf2 / Cenexin and the retinoblastoma protein. Splice variant 13.8NC, characterized in part 3.2, was analyzed in silico by an amino acid sequence motif search revealing putative binding to the retinoblastoma protein.

I was able to demonstrate a direct interaction between Rb and Odf2 / Cenexin and a functional relationship between both.

Status: in progress

Running title: A Centrosomal Protein Gets in Focus of Cell Cycle Progression – Impacting a Key Regulatory Function: Expression of the Retinoblastoma Protein is Influenced by Odf2 / Cenexin Interaction Mediated by its LxCxE Peptide Motif.

A Centrosomal Protein Gets in Focus of Cell Cycle Progression – Impacting a Key Regulatory Function: Expression of the Retinoblastoma Protein is Influenced by Odf2 / Cenexin Interaction Mediated by its LxCxE Peptide Motif.

Abstract

Odf2 / Cenexin is described as a centrosomal protein associated with the distal and subdistal appendages of the mature centriole. In somatic cells it is targeted through a N-terminal stretch of 42 amino acids to the centrosome. Recent studies established a connection of Odf2 / Cenexin to cell cycle progression by an interaction with Plk1. A well - defined key regulator of cell cycle progression is the retinoblastoma protein (Rb) a member of the “pocket protein” family. The Rb protein contains a site that recognizes and binds the LxCxE peptide motif of target proteins. Here I report the identification of Odf2 / Cenexin as a LxCxE motif bearing protein, and the interaction between Odf2 / Cenexin and Rb. Moreover, I was able to show a relationship between the expression level of Odf2 / Cenexin and its influence on Rb expression.

Introduction

An important key regulator of the mammalian cell cycle is the retinoblastoma protein (Rb). It belongs to the family of so-called pocket proteins (other members being p107 and p130) (Wang et al., 1994). Rb is characterized as a 928-amino acid protein. The small Rb pocket comprises the A and B cyclin – like domains (A cyclin – like 379 – 578 and B 642 – 791, respectively) (Horowitz et al., 1989; Onadim et al., 1992; Yandell et al., 1989). The small pocket of Rb binds LxCxE – like – sequence – containing proteins over the LxCxE binding cleft (Lee and Cho, 2002) and also includes a primary binding site for E2Fs (Flemington et al., 1993; Helin et al., 1992; Mundle and Saberwal, 2003). The large pocket region of Rb (amino acids 379 – 928) is known to have additional binding to E2Fs and is necessary for binding to other cellular proteins (Cobrinik, 2005; Xiao et al., 1995).

Rb acts as checkpoint protein in G1 / S transition preventing the cell from replication of damaged DNA (Grana et al., 1998; Taya, 1997) and has the ability to arrest cells at the G1 phase by suppressing the activity of the E2F family of transcription factors (Knudsen et al., 1998). Non – phosphorylated Rb binds E2Fs and suppresses cell cycle progression (Cobrinik, 1996; Harbour and Dean, 2000). Rb gets phosphorylated in late G1 by cyclin D - cdk4/6. That causes the cell to traverse the G1 checkpoint and enter S-phase by activation of

E2F target genes like cyclin E (Sherr and Roberts, 1999). During M / G1 transition Rb gets dephosphorylated by Protein Phosphatase 1 (PP1) and acts again as suppressor of cell cycle progression (Alberts et al., 1993). The phosphorylation of Rb by cyclin D - cdk4/6 is mediated through binding of the G1 cyclin – cdk to Rb through recognition of the LxCxE motif found in D – cyclins (Dahiya et al., 2000; Pan et al., 2001; Radulescu, 1995).

Another kinase linked to cell cycle progression and various mitotic events is Polo-like kinase 1 (Plk1). Plk1 has been described to phosphorylate cyclin B and a cohesin subunit during mitosis (Descombes and Nigg, 1998; Nigg, 1998). Recent studies described Plk1 as an interaction partner of Odf2 / Cenexin. Odf2 / Cenexin RNAi in human cells triggers Plk1 delocalization from centrosomes followed by chromosome segregation defects (Soung et al., 2006).

Odf2 first has been described as a testicular protein that stabilize the mammalian sperm tail through the formation of the outer dense fibers (Brohmann et al., 1997; Hoyer-Fender et al., 1998; Petersen et al., 1999; Turner et al., 1997). Lately it becomes more and more linked to cell cycle progression. Its cell cycle dependent centrosomal localization has been described (Lange and Gull, 1995), as well as it's vital role in the formation of primary cilia. Odf2 and Cenexin first have been identified as centrosomal proteins associated with the distal and subdistal appendages of the mature centriole (Nakagawa et al., 2001). Alternative splicing gives either rise to Odf2 or Cenexin (Huber and Hoyer-Fender, 2007; Soung et al., 2006). Cenexin is characterized by a N-terminal insertion of 42 amino acids encoded by exon 3b whereas in Odf2 variants this sequence is missing. This amino acid stretch is necessary to localize the protein to the centriole or the basal body of primary cilia (Huber et al., 2008). Cenexin deficient cells have been described to fail to undergo primary cilia formation and display a lack of the centriolar appendages (Ishikawa et al., 2005). The C-terminal stretch described by Soung *et al* seems to be required for Plk1 recruitment and therefore cell cycle progression.

The centrosome itself seems to play an important role in the regulation of cell cycle progression. First it has been described as the main microtubule organization centre of animal cells localized near the nucleus (Bornens et al., 1987). During interphase most of the cell's microtubules (MTs) are nucleated based on γ-tubulin ring complexes (γTuRCs). The γTuRCs are anchored in the pericentriolar material surrounding both centrioles of the centrosome (Dictenberg et al., 1998). The centrioles are positioned orthogonally to each other and are characterized by the appendage proteins of the mature centriole (Bornens et al., 1987). During the cell cycle the centrosome is duplicated in a semi-conservative manner and forms in the two poles of the mitotic spindle (Kochanski and Borisy, 1990).

Here I report a link between Rb as a key regulator of cell cycle progression and the centrosomal protein Odf2 / Cenexin. A known property of the retinoblastoma protein is its ability to interact with proteins that contain an LxCxE peptide sequence motif. My results demonstrate an interaction of Rb and Odf2 / Cenexin by means of biochemical and cell biological methods.

I was able to narrow down the interaction to the LxCxE cleft of Rb. Rb constructs with mutations in the LxCxE cleft, the A, and the B pocket were used to discover and confirm the LxCxE mediated interaction. Reporter gene assays excluded a functional role of Odf2 / Cenexin in Rb's function as a tumor suppressor through inhibition of E2F transcription factors and thus arresting cells at the G1 phase. The interaction between Odf2 / Cenexin and Rb reveals a novel, E2F – independent function of Rb and a novel functional aspect of Odf2 / Cenexin in cell cycle progression. Moreover a functional relationship between Odf2 / Cenexin and Rb could be shown in NIH3T3 cells. The protein levels of Rb determined by western blot analyses were affected by the physical interaction. In cells overexpressing Odf2 / Cenexin an increased Rb level could be detected while Odf2 / Cenexin RNAi leads to a decrease of Rb. To date Odf2 / Cenexin has not been identified as a protein involved directly in regulating cell cycle progression through orchestrating cell cycle checkpoint events. The described interaction with Rb functionally links Odf2 / Cenexin to more than just an appendage protein of the centrosome or a stabilizer of ciliogenesis: I propose that Odf2 is a centrosomal component required for orchestrating mitotic events through a direct interaction with Rb.

Material and methods

In silico motif search

To identify different binding motifs by ELM (*The Eukaryotic Linear Motif Resource for Functional Sites in Proteins;* http://elm.eu.org/links.html) the amino acid sequence of 13.8NC was used.

Cell culture, plasmids, transfections, RNAi and antibodies

NIH3T3 were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10 % (v/v) fetal bovine serum (FBS), 1000 U/mL penicillin, 1000 µg/mL streptomycin, and 20 mM L-Glutamine at 37°C and 5% CO_2.

13.8NC-GFP (Huber et al., 2008) was subloned using EcoRI and NotI in pET28a(+) named p13.8NC-6His.The p13.8NCΔgfp construct was designed by SmaI and NotI restriction of p13.8NC-gfp. Other Odf2 expression constructs used are further described (Donkor et al., 2004) or kindly provided by Kyung S. Lee (pShutttleEGFP_hCenexin (Soung et al., 2006)).

The full length Rb (pSGSL SV40HA-Rb) was a kind gift from U.M. Bauer. pGEX-Rb coding a GST fusion protein of aa 379 – 928 (pocket domain of Rb formed of aa 379 – 578) was kindly provided by U. K. Binné (Binne et al., 2007). Rb constructs mutated in the pocket domain coding region (pCMV-Rb9, pCMV-Rb36, pCMV-Rb63) also kindly provided by U.M. Bauer (Dick and Dyson, 2002; Dick et al., 2000).

For reporter gene assays phRL-SV40 (Promega, Mannheim, Germany), and pCMV HA E2F1, pGL3 basic TATA 6xE2F kindly supplied by K. Helin were used.

Plasmid transfections were done using Transfectin (BioRad, Munich, Germany) 24 h before fixation or biochemical analyses. Indirect immunostaining was carried out as previously described (Huber et al., 2008). Mouse anti HA antibody (clone HA.C5, abcam, Cambridge, UK) was used.

RNAi experiments were carried out with *stealth* siRNA (ODF2MSS207236, Invitrogen, Karlsruhe, Germany) and siLentFect reagent (BioRad, Munich, Germany). Samples were analyzed 48 h after transfection.

Pull down experiment

E. coli BL21 (p13.8NC-6His, pGEX-Rb) was pre - cultured in 5 mL LB- medium at 37 °C. Expression cultures were inoculated with preculture 1:500 and expression was induced by auto induction in ZYM 5052 o/n at 37°C (Studier, 2005). Induction of pGEX-Rb resulted in a soluble 87 kDa protein. The inclusion bodies of the 6His-13.8NC fusion protein were dissolved in wash buffer (50 mM Tris-Cl pH 7.5, 100 mM NaCl, 1 mM EDTA, 0.2 mM PMSF, and 1 mM DTT) and purified by three repetitive sonication and centrifugation steps. Additionally pellet was two times washed and sonicated with 1% Triton – X – 100 (v/v) containing wash buffer. The pellet was dissolved in 100 mM Tris-Cl pH 8.0, 50 mM Glycine and 8.5 M Urea. 5 mM GSSH, and 0.5 mM GSSG were added and stirred o/n at 4°C. Refolding of the protein was done by dialyzes against Refolding buffer pH 8,0 (100 mM Tris-Cl pH 8.0, 400 mM L-Arginine, 1mM EDTA, and 0.2 mM PMSF) with decreasing urea concentrations over several days at 4°C (4 M, 2 M, 1 M, and 0,5 M Urea). The penultimate dialyzing step was performed against refolding buffer without Urea, the last one against 1 x PBS pH 7.6. Each dialyzes was performed o/n at 4°C. 6His-13.8NC fusion protein was quantified by BSA standard via Coomassie staining.

For in vitro interaction 6His-13.8NC was added to washed Ni-NTA agarose (Wash buffer pH 7.6: 50 mM NaPi, 500 mM NaCl, 30 mM Imidazole, 0.2 mM PMSF and Protease Inhibitor (Protease Inhibitor Cocktail Tablets, complete Mini, Roche, Mannheim, Germany)) and incubated 2 h at 4°C and constant agitation. GST - Rb fusion protein was added to the resin and incubated at least 2 h at 4 °C. Resin was washed 4 times and last washing step was performed o/n.

Elution was done with 50 mM NaPi, 500 mM NaCl, 500 mM Imidazole, 0.2 mM PMSF and analyzed by SDS - PAGE and Western blotting. Membrane were probed against goat anti GST (Promega, Mannheim, Germany) and mouse anti His tag antibodies (abcam, Cambridge, UK).

Co-Immunoprecipitation

Alternatively NIH3T3 cells were transfected with the full length Rb or Rb mutated constructs (pSGSL SV40HA-Rb, pCMV-Rb9, pCMV-Rb36, pCMV-Rb63) using Transfectin reagent (BioRad, Munich, Germany). Cells were lysed in Lyses Buffer (1 x PBS containing 1 % Nonidet P40, 100 µg/ mL (w/v) PMSF and protease inhibitor mix (Protease Inhibitor Cocktail Tablets, complete Mini, Roche, Mannheim, Germany)) using 1 mL buffer per 10^7 cells. After incubation for 20 min on ice with occasional mixing the lysate was disrupted by passing it at least 10 times through a syringe with a 21 – gauge needle. After centrifugation monoclonal mouse anti HA antibody (12CA5) was added to the supernatant and incubated 2 h at RT. Immobilized protein G agarose (Thermo Scientific, Karlsruhe, Germany) was washed twice with lyses buffer and added to supernatant. Incubation was carried out o/n at 4°C. Resin was washed five times and an equal volume of electrophoresis sample buffer was added. Proteins were extracted from the beads by heating to 95 °C for 5 min. Proteins were finally analyzed by SDS - PAGE and immunoblotting using rabbit anti-Odf2 (Hoyer-Fender et al., 2003) and anti – HA tag (12CA5) antibodies.

Reporter Gene Assay

Cells were transfected in 12 well dishes with the firefly luciferase reporter gene plasmid pGL3 basic TATA 6xE2F using 1.5 µg / well. 100 ng / well 13.8NC-GFP, pSGSL SV40HA-Rb, and pCMV HA E2F1, or *stealth* siRNA (ODF2MSS207236, Invitrogen, Karlsruhe, Germany) were optionally cotransfected. To normalize for transfection efficiency 100 ng/well of phRL-SV40 were cotransfected. After 24 h cells were lysed in Luciferase-Lyses buffer (150 mM Hepes, 0.25 % Triton-X-100 in ddH_2O) and analyzed with the Dual-Glow Luciferase Assay System using a Berthold luminometer.

Proliferation Assay

Cells were seeded in flat bottom 96 well plates. Adherent cells were transfected with siRNA or plasmid DNA. The viability of the cells was measured after 24 h, 48 h, 72 h, and 96 h using Alamar blue reagent (Biosource, Invitrogen, Karlsruhe, Germany).

Immunoblotting analyses

NIH3T3 cells were cultured as described above and transfected with the various constructs or siRNAs as described above. After 24 h (overexpression analyses) or 48 h (RNAi) cells were trypsinized, rinsed with PBS, and harvested by centrifugation. Cell lysates were prepared by adding an equal volume of electrophoresis sample buffer (in 1 x PBS) and heating to 95 °C for 10 min.

An equal amount of cells was loaded on SDS - PAGE and analyzed by immunoblotting using mouse anti-GFP (abcam, Cambridge, UK), mouse anti-retinoblastoma (mAB245; Millipore, Schwalbach/Ts., Germany), rabbit anti cyclin D1 (abcam, Cambridge, UK), and mouse anti alpha tubulin (Oncogene research products, Merck, Darmstadt, Germany) antibodies.

Results

Identification of Odf2 / Cenexin as LxCxE binding motif protein

The amino acid sequence of Odf2 / Cenexin (13.8NC) was used to determine putative binding motifs using the *Eukaryotic Linear Motif Resource for Functional Sites in Proteins* (http://elm.eu.org/links.html). At position 392 – 396 the LxCxE motif was detected, specifically encoded by the QLRCKEA sequence (see figure 1). LxCxE motif containing proteins often interact with the small pocket domain of the Rb protein through its LxCxE cleft. The determining triplet leucine, cysteine, and glutamate provide the specificity for binding but high affinity binding requires other flanking residue interactions. It has been described that the amino acid side chains in front of the leucine (here glutamine), the one following the glutamate (here alanine) and the third behind the glutamate (here asparagine) should not be positively charged to enable tight binding to Rb. The second amino acid behind the glutamate (here a glutamate) should preferably be a hydrophobic residue (Singh et al., 2005).

The flanking amino acids in Odf2 / Cenexin are neutral and therefore do not weaken the binding of Odf2 / Cenexin and Rb. The hydrophilic – charged glutamate should not modulate the strength of the interaction negatively.

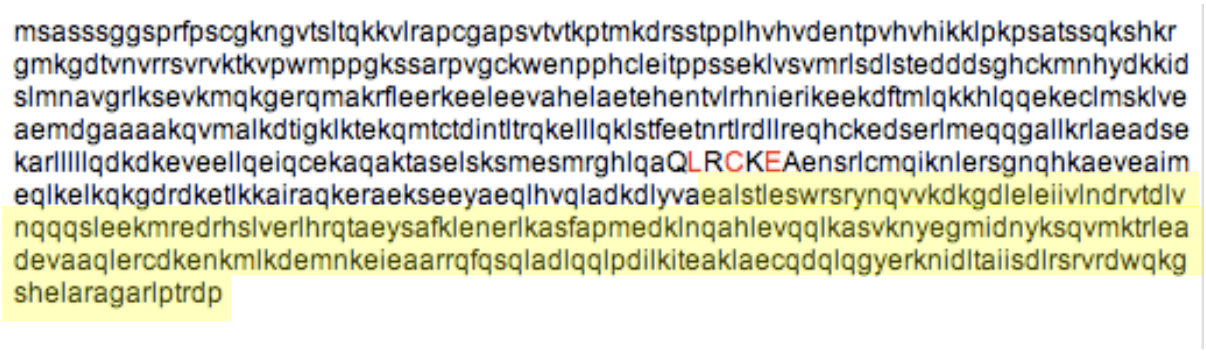


Figure 1: Localization of the LxCxE motif in Odf2 / Cenexin. Conserved amino acids of the retinoblastoma ligand binding motif are shown in red. Shown is the amino acid sequence of 13.8NC that was fused to EGFP for expression analyses (for cloning strategy see (Huber et al., 2008). Odf2N2C-gfp results in a truncated GFP - Odf2 fusion protein without the LxCxE motif (sequence highlighted in yellow; for cloning schema see (Donkor et al., 2004).

Odf2 / Cenexin interacts directly with the non phosphorylated form of the retinoblastoma protein

The *in silico* predicted interaction between Odf2 / Cenexin and the retinoblastoma protein was experimentally tested by His pull-down experiments using recombinant expressed proteins.

Expression of 13.8NC - 6His resulted in a 85 kDa Odf2 / Cenexin fusion protein (Fig. 2a). The truncated retinoblastoma protein encodes the amino acids 379 – 928 of the full-length protein and therefore includes the A cyclin like domain of the small pocket (379 – 578 aa). Expression resulted in a soluble ~87 kDa GST fusion protein (Fig. 2b (Binne et al., 2007). Protein concentrations were determined by Coomassie staining and BSA standard series and equal amounts of proteins used for the in vitro binding experiment. The predicted interaction of Odf2 / Cenexin and the Rb protein could clearly be demonstrated. The GST – Rb fusion protein was detected by anti GST antibodies in the first elution. However the bulk was still bound to a Ni-NTA agarose resin through the His – tagged Odf2 / Cenexin. Odf2 / Cenexin was detected by anti His - tag antibodies in the eluted fraction as well as in the bead bound fraction. The results show a direct interaction of the non-phosphorylated form of the retinoblastoma protein and Odf2 / Cenexin in pull down experiments (Fig. 2c).

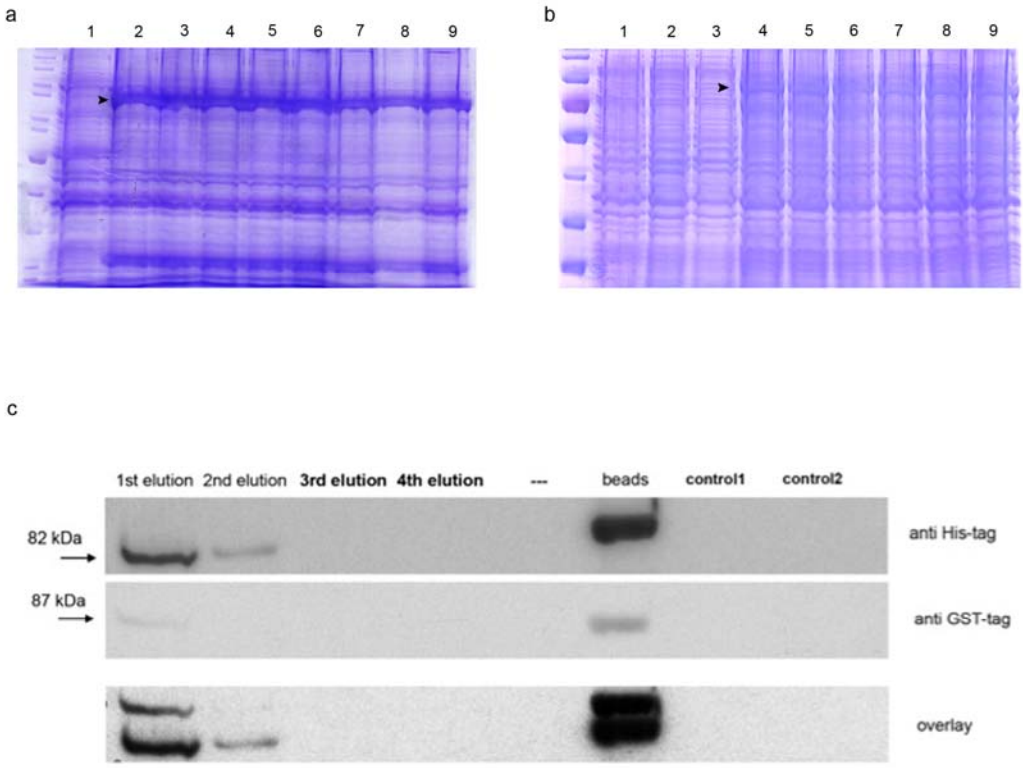


Figure 2: His pull down experiment with recombinant His - Odf2 / Cenexin and the GST –tagged retinoblastoma protein. **a**. Coomassie staining of the SDS - PAGE shows a ~ 85 kDa Odf2 / Cenexin His fusion protein (marked by an arrow) Eight clones were tested (lane 2 – 9) in comparison the extract of a non induced clone was loaded in lane 1. **b**. Induction of the GST-Rb fusion construct resulted in a ~87 kDa protein (marked by an arrow, lane 4 - 9) in comparison extract of a non-induced culture was loaded (lane 1 – 3). **c**. Pull down of GST-Rb with Odf2 / Cenexin His using Ni –NTA. Odf2 was bound to the Ni - NTA resin and could been detected with the anti – His tag antibody in eluate 1 and 2 as well as the boiled beads. In the same eluate also Rb-GST could be detected with an antibody against the GST - tag. The last wash buffer before elution was loaded as control1. Rb-GST incubated with the Ni-NTA was loaded as control 2, to exclude unspecific binding of Rb-GST to the resin.

To obtain further information about interaction of Odf2 / Cenexin and Rb immunoprecipitation was performed using NIH3T3 cell lysates transiently expressing the full length Rb - HA. Anti HA antibody immunoprecipitates were examined by western blot analysis with polyclonal antibodies against Odf2, and monoclonal antibodies against the Rb - HA tag. Untransfected cells were used as control. The interaction between Rb and Odf2 could clearly be demonstrated (Fig. 3b). To determine the significance of the interaction mediated by the Odf2 / Cenexin LxCxE motif Rb - HA fusion proteins were mutated in the small pocket domain and transiently expressed. Immunoprecipitation was performed with anti HA antibodies (Tab. 1).

Precipitates were immunoblotted using anti Odf2 antibodies. Interaction of Odf2 and Rb could also be demonstrated with Rb36-HA and Rb63-HA mutants. No Odf2 was precipitated with the Rb9-HA mutant. This mutant was designed with the goal to disrupt the hydrophobic pockets that are occupied by the leucine and cysteine residues of the LxCxE motif. In the

Rb9 mutant the sides of this hydrophobic cleft are removed. Isoleucine at position 753, asparagine at position 757, and methionine at position 761 are substituted against alanine (I753A, N757A, M761A, (Dick et al., 2000). This is predicted to make the leucine and cystine residues a poor fit for this hydrophobic groove.

Table 1: Retinoblastoma mutants used in this study, accompanied by the corresponding changes that have been incorporated into Rb. Substitutions are listed concerning the full-length amino acid sequence of Rb. (For comparative alignment of amino acid sequences see appendix A).

Construct Name	Region	Substitution
pCMV-Rb9	LxCxE cleft	I753A, N757A, M761A
pCMV-Rb36	N – terminal domain	F132S
pCMV-Rb63	A – cyclin like domain	S463A, E464A, R544A, K548A
	B – cyclin like domain	K652A, R656A, L660A, T664A, R668A
	Large pocket region	K873A, K874A

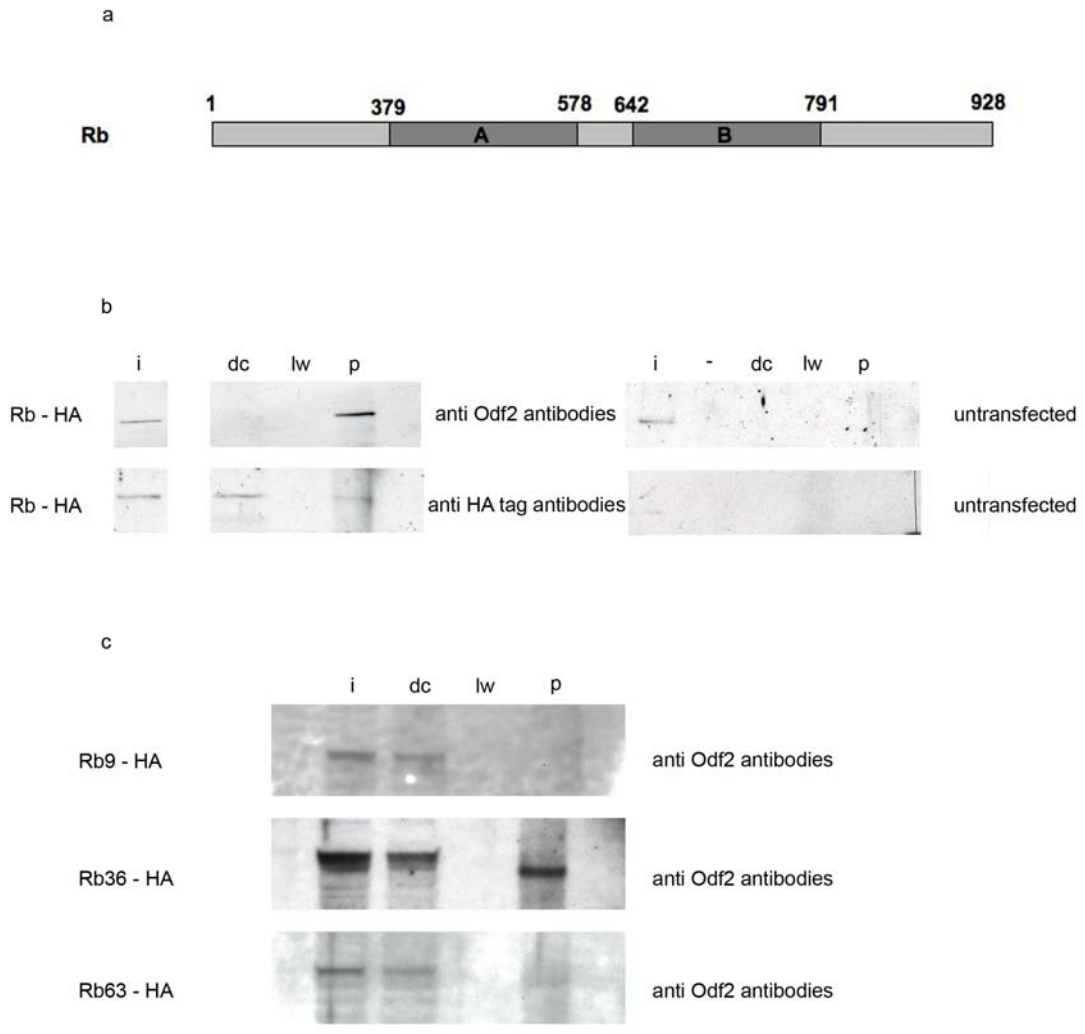


Figure 3: Co-Immunoprecipitation. **a**. The domains of the full length Rb. The A and B cyclin like domain form the small pocket region (379 – 791 aa), the large pocket domain is annotated from aa 379 to 928. The hydrophobic LxCxE cleft localizes between aa 753 and 761. **b**. The full length Rb was transient overexpressed in NIH3T3 cells and the extract was prepared 24 h after transfection. Immunoprecipitation was performed with α - HA antibodies and the precipitates were immunoblotted using α-Odf2 antibodies and α-HA antibodies (left). As control extract of untransfected NIH3T3 cells was prepared and precipitated with α - HA. I, input; dc, depletion control after incubation with immobilized protein G; lw, last wash buffer; p, precipitate. **c**. Rb mutants were transiently expressed and immunoprecipitation was performed after 24 h with α - HA antibodies. The immunoblotted precipitates were analyzed by probing against Odf2. I, input; dc, depletion control after incubation with immobilized protein G; lw, last wash buffer; p, precipitate. No Odf2 could be precipitated with the Rb9, a LxCxE cleft mutant.

Odf2 / Cenexin interaction to Rb does not impact the activity of E2F target genes and has no influence on cell proliferation

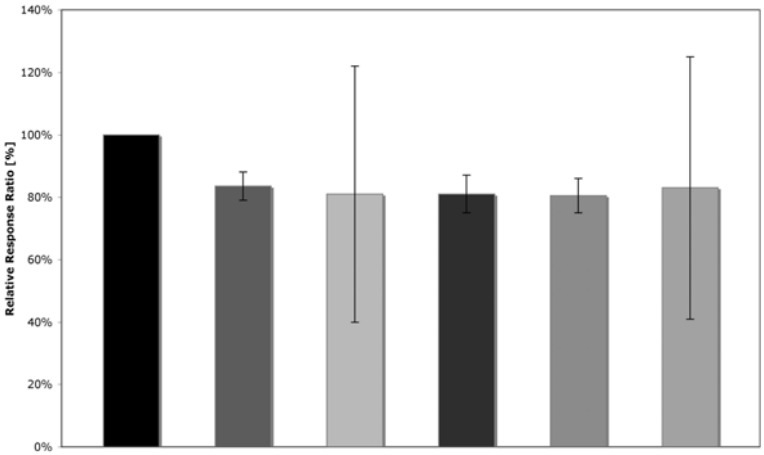
To characterize the function of Odf2 / Cenexin in cellular context I was interested to address how an overexpression or a knock down of Odf2 influences E2F related cell proliferation. The inactive, hyperphosphorylated form of Rb appears when cells enter S – phase and is dominant in G2 and M phase. The active, underphosphorylated forms of Rb dominate in G1 (Buchkovich et al., 1989). Cyclin – dependent kinase mediated phosphorylation thus controls and regulates the growth – suppressive activity of Rb (Hinds et al., 1992). Underphosphorylated Rb can bind to E2Fs inhibit their transactivation function (Hiebert et al., 1992) thus converting E2Fs to an active repressor of transcription.

To determine the transactivation function of E2F in Odf2 / Cenexin overexpression or knock down background I measured the enzymatic activity of the luciferase protein transcribed from a reportergene plasmid containing a six fold E2F TATA motif. Cells transfected with the pSGSL SV40 HA-Rb show a decrease of activity as expected compared to the control (Fig. 4). The control was transfected with the reportergene plasmid, an E2F expression plasmid, and the *Renilla* luciferase plasmid to calculate the relative response ratio. The overexpression of Rb leads to a binding of (also overexpressed) E2F and a lower transcription rate of the *Firefly* luciferase gene driven by the six fold E2F TATA sequence. The luciferase mRNA level is directly proportional to the luciferase protein level and therefore the influence of Odf2 / Cenexin on transcription can directly measured through changes in substrate conversion.

No differences in the activity were measured after Odf2 / Cenexin overexpression or knock down. Co-transfected cells expressing Odf2 / Cenexin and Rb also displayed no increase in E2F promoter transactivation. The same holds true for Odf2 / Cenexin RNAi and overexpression of Rb. In summary the interaction of Odf2 / Cenexin with Rb does not effects the growth – inhibitory effects through the regulation of the E2F family of transcription factors, which are required for the expression of genes involved in G1 – S transition and DNA

replication. The physically interaction of Rb with Odf2 / Cenexin reveals an E2F – independent function of Rb.

To confirm these findings a proliferation assay using the alamar blue method was performed. I compared the viability and proliferation kinetic of Odf2 / Cenexin overexpressing, RNAi, and untreated control cells over a period of 72 h (Fig.5, above). Rb and Odf2 / Cenexin co-transfected cells and Odf2 RNAi cells overexpressing Rb were also analyzed (Fig. 5, below).



pGL3basic TATA 6xE2F	x	x	x	x	x	x
phRL	x	x	x	x	x	x
pCMV HA E2F	x	x	x	x	x	x
pSGSL SV40 HA - Rb				x	x	x
p13.8NC-gfp		x			x	
siRNA			x			x

Figure 4: Reportergene assay to determine the effects of the transactivation function of E2F. All cells were transfected with the reporter plasmid pGL3basic TATA 6xE2F, pCMV HA E2F and the *Renilla* luciferase coding construct phRL for normalization. Control is set 100 %. Shown is the relative response ratio (*Renilla* luminescence / *Firefly* luminescence as % of untreated sample). Transfection of pSGSL Sv40 HA – Rb leads to decrease of reporter activity as expected. Odf2 / Cenexin overexpression as well as RNAi don't influenced the reporter activity and therefore the transactivation function of E2F. No changes after co-transfection of siRNA or Odf2 / Cenexin expression constructs and Rb could be detected. (For raw data rather measured values see appendix, appendix B)

The overexpression of the different proteins did not affect the proliferation ratio and viability of the examined cells. 72 h after transfection almost the same conversion of the measured alamar blue reduction could be observed in control and transfected cells. Compared to the untreated control cells there is no significant difference in cell growth and viability. However, changes in growth kinetic could be observed. Focused on the kinetic between 24 h and 48 h after transfection of Odf2 / Cenexin containing the LxCxE peptide motif (p13.8NC-gfp, p13.8NCΔgfp) the kinetik of these cells nearly showed no proliferation activity while reduction of the alamar blue reagent in control cells was increased (Fig.5). RNAi cells did not show this

arrest between 24 h and 48 h after transfection. They grew weakly but constant. Similarly effects, in terms of the decrease of proliferation activity and consequently of measured values were observed in cells treated only with the transfection reagents siLentFect or Transfectin (for measured values see appendix C).

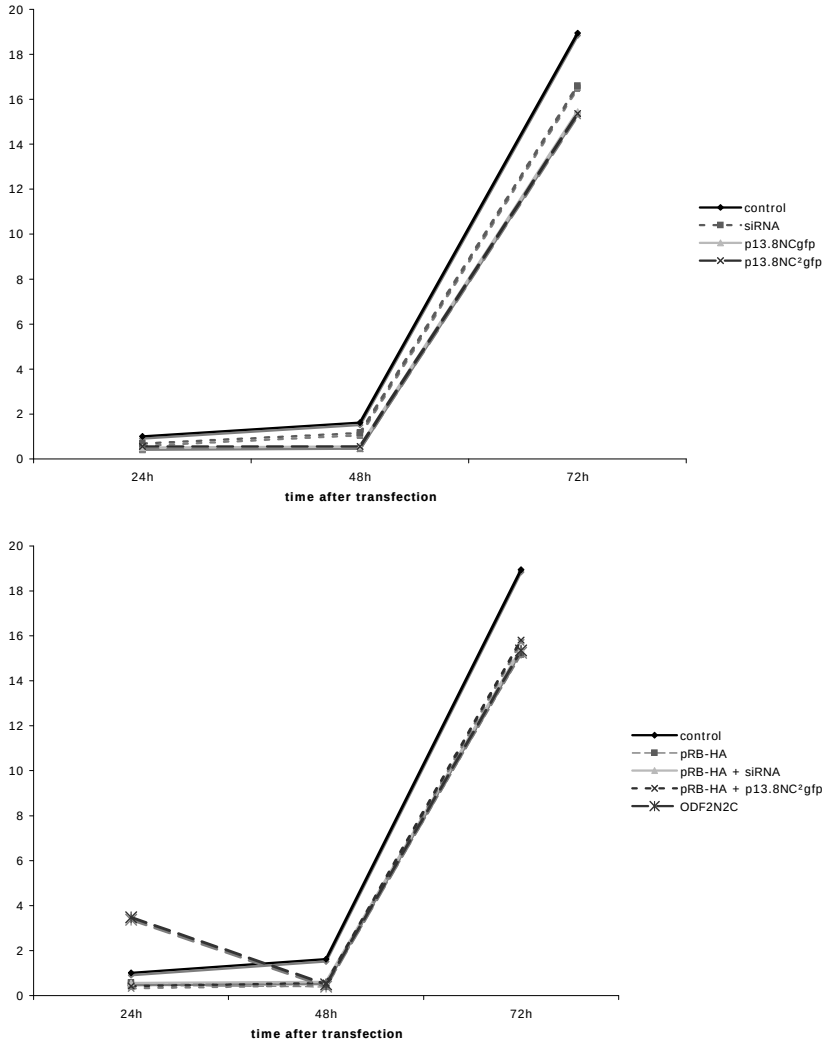


Figure 5: Growth kinetic and viability determined by Alamar Blue reagent. Average of ΔOD values measured from two representative experiments of 6 – 12 individual experiments. Control at time point 24 h after transfection was set to 1. Other values normalized to control. Shown is the comparison of Odf2 / Cenexin overexpressing and RNAi cells. The in this study used gfp – Odf2 / Cenexin fusion construct (p13.8NC-gfp) is examined alike a construct without gfp to exclude growth effects by GFP. Equal growth kinetic was observed after transfection of pEGFP-N1 (data not shown). All probes analyzed show equal kinetic and proliferation ratio. Interestingly the growth of control cells is slightly accelerated whereas cells overexpressing the full length Odf2 / Cenexin show nearly constant growth behavior. RNAi cells grow comparable to control cells. No variances in growth kinetics and viability could be determined in Rb overexpressing cells or Odf2 / Cenexin and Rb co-transfected cells (below).

Rb overexpressing cells or cells co – transected with Rb and Odf2 / Cenexin showed similar behavior. A proliferation arrest between 24 h and 48 h after transfections takes place (Fig.5, above).

In summary Odf2 / Cenexin does not effect the growth – suppressive activity of Rb by regulation of the E2F transcription factors, which are required for the expression of genes involved in G1 – S transition and DNA replication and therefore cell cycle progression. However Odf2 / Cenexin overexpressing cells as well as RNAi cells seems to be arrested and their growth kinetic is influenced.

Overexpression of Odf2 constructs influence the endogenous Rb expression level in NIH3T3 cells

To investigate effects on Rb after Odf2 / Cenexin overexpression and knock down exponentially growing cultures were transfected with Cenexin constructs or Odf2 / Cenexin siRNAs. 24 h later the Rb level of the transfected cells and untreated control cells was analyzed by immunoblotting (Fig.6).

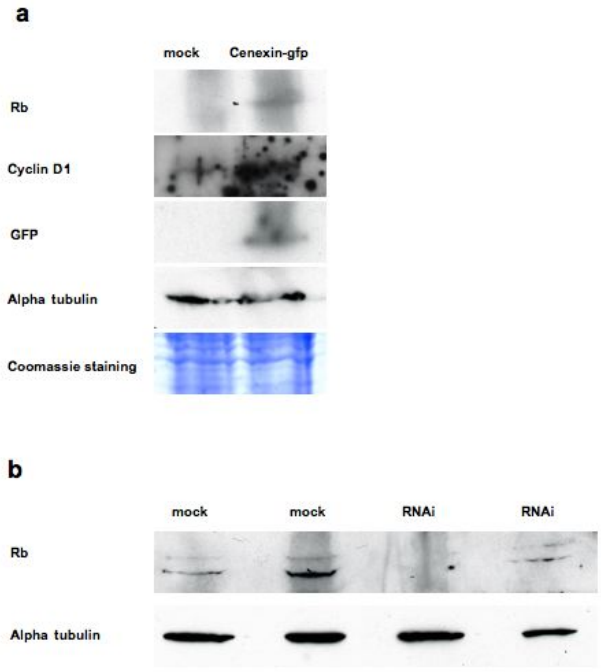


Figure 6: Effects on Rb after Odf2 / Cenexin overexpression or RNAi. a. Cells overexpressing Cenexin (transfected with pShuttleEGFP_hCenexin, kindly provided by Kyung S. Lee) and untreated control (mock) were harvested and counted. Equal volumes of cells (10^5 cells) were loaded and analyzed by immunoblotting with antibodies against Rb, Cyclin D1, GFP, and alpha tubulin. The overexpression of Cenexin had an effect on the Rb level and Cyclin D1 amount. b. Analyses of Odf2 RNAi cells by western blotting. Membrane was incubated with anti Rb and anti alpha tubulin antibodies. In RNAi cells a decreased Rb amount is detected (10^7 cells). This correlates with the increase of Rb level after Cenexin overexpression.

The Cenexin – GFP expression was proved by western blotting against GFP. Alpha tubulin was used as loading control. Interestingly an increase of Rb expression was determined in Cenexin overexpressing cells, while a decrease of Rb was measured in RNAi cells. Surprisingly the overall protein levels of the non-phosphorylated form of Rb (detected by the

antibody) were directly influenced by Odf2 / Cenexin overexpression. Moreover an increase of cyclin D1 could be detected in Odf2 / Cenexin overexpressing cells.
The overexpression of Cenexin results in an enrichment of non-phosphorylated Rb and cyclin D1 in exponentially growing cell cultures.

Intracellular localization of Rb depends on Odf2 / Cenexin overexpression and RNAi: RNAi and overexpression release Rb from heterochromatin

To examine the localization of Rb and a possible effect of Odf2 / Cenexin overexpression or RNAi NIH3T3 cells were transfected with pSGSL SV40HA – Rb and visualized by indirect immunofluorescence using anti HA – antibodies.
In an exponentially growing cellculture Rb is localized to heterochromatic regions in the nucleus (Fig. 7 a – c) with diffuse nucleoplasmic staining and exclusion from nucleolar regions (Fig. 7 d). It has been described that the Rb protein is always nuclear distributed, but varies in its localization. The Rb protein is dispersed throughout nuclei during early and mid S – phase, but becomes concentrated in nucleoli in late S or G2 phase (Takemura et al., 2002).
In cells overexpressing Odf2 / Cenexin which normally localizes to the centrosome Rb is not detectable on heterochromatin. All Rb is diffuse distributed in the nucleus and the nucleoli are spared (Fig. 7 e – f). The same intracellular localization can be observed in RNAi cells. Exponentially growing Odf2 / Cenexin RNAi cultures also lose the heterochromatin associated localization of Rb and the diffuse nuclear localization of Rb (Fig. 7 g). The observed localization of Rb indicates that the examined cells are in early or mid S – phase.
Interestingly the transfected Odf2N2C – gfp construct that encodes the truncated Odf2 without the LxCxE peptide motif did not show a similar localization of Rb in comparison to control cells (Fig. 7 h – i). These cells show a smooth Odf2 - GFP distribution in the cytoplasm and only weak centrosomal localization. In these cells the Rb is localized in the nucleus with the same homogenous distribution that was observed in RNAi cells and p13.8NC – gfp transfected cells.

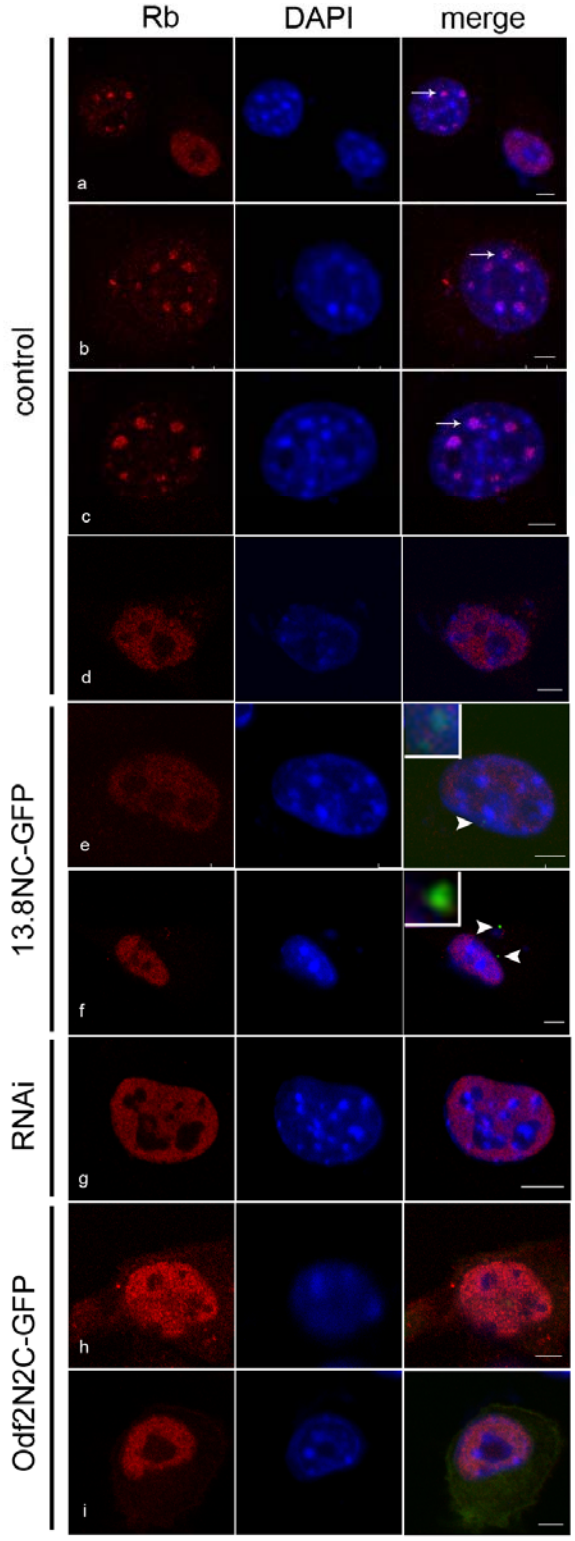


Figure 7: Intracellular localization of HA – tagged Rb protein, detected by indirect immunofluorescence using an HA – antibody (bars 5 µm). In control cells Rb is either localized homogenously distributed in the nucleoplasm (d) or associated with the heterochromatic regions also stained with DAPI (marked by arrows, a – c). In Odf2 / Cenexin overexpressing cells (centrosomal Odf2 marked by arrowheads) as well as in RNAi cells no heterochromatic localization can be observed. All Rb is detected in the nucleoplasm sparing the nucleoli of the cells.

Discussion

In my work I identified the physical interaction of the retinoblastoma protein and Odf2 / Cenexin and narrowed down the interaction to the LxCxE binding cleft on the surface of Rb. Using Rb constructs with mutations in the LxCxE cleft, the A, and the B pocket I analyzed and confirmed the LxCxE mediated interaction. This binding site is utilized by viral oncoproteins to inactivate Rb, but is also the binding site for a variety of cellular proteins. Multiple functions of Rb (e.g. cell proliferation, cell cycle checkpoint control, cell differentiation) require an interaction directly or indirectly (through multiple protein complexes). The interaction with cellular proteins can be modulated in strength through, for example, posttranslational modifications or by the primary amino acid sequence present in binding sequences. It has been described that positively charged amino acids that flank the LxCxE peptide sequence do not abolish binding, but weaken binding in differing extents. This results in the association and exchange of Rb with different protein complexes (Singh et al., 2005). The Odf2 / Cenexin LxCxE motif is flanked by neutrally charged amino acids and the binding therefore should be of higher affinity, even though the second amino acid behind the LxCxE peptide motif is not hydrophobic, which would mediate the strongest binding (see figure 1). For this study recombinant expressed unmodified Odf2 / Cenexin and Rb was used. Interaction studies with posttranslationally modified proteins were not carried out. The physical interaction was demonstrated by pull down experiments. A stabilization of binding by posttranslational modifications of Odf2 / Cenexin couldn't be excluded, but they does not seem to be necessary. In summary Odf2 / Cenexin interacts with the underphosphorylated form of Rb through the LxCxE peptide motif and stably binds to Rb due to the flanking amino acids.

Interestingly Odf2 / Cenexin compete with cyclin D1 for the LxCxE binding cleft on underphosphorylated Rb. Rb gets phosphorylated in late G1 by cyclin D – cdk 4/6. This causes the cell to traverse the G1 checkpoint and enter S – phase (Sherr and Roberts, 1999). The effect of the Odf2 / Cenexin – Rb interaction on cell cycle progression was shown by cell growth kinetics with the Alamar blue assay. The overexpression of Odf2 / Cenexin seems to cause a proliferation arrest. This may be due to a masking effect of the LxCxE cleft on the Rb surface by Odf2 / Cenexin. As a consequence the activation of Rb is blocked and no G1/S checkpoint transition can occur. Therefore an accumulation of nonphosphorylated Rb and increased cyclin D1 levels were detected by western blot analyses. In contrast RNAi of Odf2 / Cenexin caused a decrease of the nonphosphorylated Rb level. The cells may be able to enter S – phase after phosphorylation of Rb by cyclin D – cdk 4/6 but can not pass the S – phase checkpoint, because the Odf2 / Cenexin RNAi leads to a degradation of Odf2 / Cenexin transcripts and therefore a prohibition of Odf2 / Cenexin protein expression.

Consequently not all components for centrosome duplication are synthesized and the cells get arrested in S – phase. To confirm these hypothesis western blot analyses of siRNA treated cells should be performed to determine the cyclin A level.

To date these results support that the physically interaction is mediated through the LxCxE binding cleft on the surface of Rb and that this binding does not effect the binding of E2Fs to the small pocket domain of Rb. Moreover it has no influences on E2Fs and their transactivation function. However E2F is the best-known Rb – associated protein, but there is very clear evidence that Rb has additional targets and functions in various cellular events.

Future experiments will have to determine the cellular function of the described interaction and dissect its role in cell cycle progression. Previous studies of Rb have focused on its role in the G0 / G1 phase of the cell cycle and the G1 – to – S transition. Far less attention has been paid to the possibility that Rb may influence progression through later stages of the cell cycle. Recent studies discuss that the LxCxE cleft is required for H4 – K20 trimethylation at pericentric heterochromatin (Isaac et al., 2006). Thus Rb does not only effect cell cycle progression through the well described transcriptional repression of E2F target genes but uses the LxCxE binding cleft to regulate chromatin structure. Interestingly mutations of the LxCxE cleft cause a defect in mitotic progression, a defect that is characterized by lagging chromosomes during anaphase. In recent studies Odf2 / Cenexin is also linked to more than being only a cytoskeletal and centrosomal scaffold protein. The characterized interaction with Plk1 links Odf2 / Cenexin to cell cycle events. It will take further investigation to connect all these findings and finally develop a model that Odf2 / Cenexin and Rb fit in. To date only the repressive effect of Odf2 / Cenexin in E2F – dependent transcription could be excluded.

Acknowledgement

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3.4. Functional characterization of Odf2 / Cenexin by RNAi mediated knock down

In this part of my thesis I describe the influence of an Odf2 / Cenexin knock down on cell cycle progression and chromatin dynamics. I was able to show an Odf2 / Cenexin dependent influence on Histone 3 Serine 10 phosphorylation and I will discuss if this is a transcriptional or mitotic event. Furthermore the chromosomal mitotic defects observed after Odf2 / Cenexin RNAi are analyzed in context of chromosome cohesin or microtubule attachment defects.

Status: in progress

Working title: Odf2 / Cenexin – A Chromatin Story Written by a Centrosomal Protein.

Odf2 / Cenexin: A Chromatin Story Written by a Centrosomal Protein

Abstract

More and more the centrosomal protein Odf2 / Cenexin steps into the focus of cell cycle progression events. First identified as a major component of the mammalian sperm tail it was later characterized as a centrosomal protein associated with the appendages of the mature centriole. For a long time no further function has been specified. First functional approaches linked Odf2 / Cenexin to ciliogenesis. Cells lacking Odf2 / Cenexin are no longer able to generate primary cilia. Next an interaction with Plk1 was described, a first hint and link to cell growth and cell cycle progression. I was able to show a direct interaction of Odf2 with a key regulator of the cell cycle, the retinoblastoma protein (Rb). This interaction is mediated by the LxCxE peptide motif of Odf2 / Cenexin and the LxCxE binding cleft of Rb. In this study I will demonstrated that a loss of Odf2 / Cenexin not only impairs the generation of primary cilia but also has impact on regulatory cellular events. Odf2 / Cenexin RNAi causes a decrease in histone 3 Serine 10 phosphorylation (H3S10ph) and leads to miss - attachment of spindle microtubules to centromeres during mitosis. Odf2 / Cenexin seems to be an important protein that prevents cells from mitotic segregation defects and therefore maintain the genetic integrity.

Introduction

Odf2 first has been identified as a major component of the mammalian sperm tail associated with the microtubules of the axonema (Brohmann et al., 1997; Hoyer-Fender et al., 1998). Further studies identified the protein associated with the major microtubule organization centre of animal cells, – the centrosome. It preferentially localizes to the distal appendages of the mature centriole and is expressed in a cell cycle dependent manner (Lange and Gull, 1995; Nakagawa et al., 2001). I was able to show, that Odf2 and Cenexin are products of one gene that differ in a short N-terminal amino acid stretch encoded by exon 3b (Huber and Hoyer-Fender, 2007). GFP fusion proteins that include this insertion are strongly associated with the centrosome, the acetylated microtubule network, and primary cilia (Huber et al., 2008). Further studies have already demonstrated an association with microtubules in general (Donkor et al., 2004). Odf2 / Cenexin has also been linked to be actively involved in cilia formation (Ishikawa et al., 2005). Odf2 deficient cells lack their distal appendages as well as the capability of primary cilia formation. Additionally a connection to cell cycle

progression by recruitment of Plk1 has been described (Soung et al., 2006). The interaction with the retinoblastoma protein (described previously (2.3)) also links Odf2 / Cenexin to cell cycle events.

A major event in cell cycle progression is the access of mitosis. At the onset of mitosis, all M-cdks of the cyclin A/B – cdk1 complexes get dephosphorylated and therefore activated. The G2/M checkpoint that has to be crossed to enter mitosis is passed (Ubersax et al., 2003). The now active complex phosphorylates many proteins acting in chromosome condensation, retraction of the nuclear envelope, assembly of the mitotic spindle, and alignment of the condensed chromosomes in the metaphase plate (for a review, see (Morgan, 1997). Chromosome condensation commences in prophase and along with that comes strong Aurora B driven phosphorylation of histone 3 serine 10 (H3S10ph) (Crosio et al., 2002; Giet and Glover, 2001; Wei et al., 1998). The post-translational modification of H3S10ph marks pericentric heterochromatin in late G2 and spreads over condensing chromosomes in mitosis. H3S10 phosphorylation also has been implicated to play a role in transcription from Polymerase II promoters (Hendzel et al., 1997). In vitro studies have shown a functional interdependence of the site-specific H3 tail modifications (H3S10 phosphorylation and H3K9 methylation) and regulation of higher order chromatin structures (Rea et al., 2000). In cells lacking the methyltransferase activity that selectively methylates lysine 9 the serine 10 phospho mark is increased and aberrant mitotic divisions are induced. Another methyl – phos switch is proposed by Fischle *et al*.. In interphase cells histone 3 lysine 9 trimethylation recruits Heterochromatin protein 1 (HP1) to distinct chromosomal regions. This recruitment is involved in the establishment of heterochromatin (Sims et al., 2003; Stewart et al., 2005), where phosphorylation of H3 is decreased. During mitosis H3K9me3 bound HP1 is ejected from chromatin through phosphorylation of H3S10. This may trigger proper chromosome condensation and segregation (Fischle et al., 2005). In mammalian cells only a fraction of HP1α, one of three HP1 isoforms, stays associated with the inner centromeric chromosome region (Schmiedeberg et al., 2004). *S.pombe* HP1 homologue *Swi6* is required for sister chromatid cohesion and correct segregation of chromosomes (Pidoux and Allshire, 2004). A similar role is suggested for mammalian HP1 (Yamagishi et al., 2008).

In eukaryotic cells a protein complex called cohesin connects sister chromatids. The cohesin complex is formed by Smc1, Smc3, Scc1, SA1 and SA2 (Guacci et al., 1997; Michaelis et al., 1997). In yeast as well as in mammalian cells the association of the cohesin complex with chromosomes depends on the loading factors Scc2 and Scc4 (Ciosk et al., 2000; Michaelis et al., 1997). The displacement of cohesin from chromosomes during prophase of mitosis is mediated by phosphorylation of the SA1 and SA2 subunits by Plk1 (Hauf et al., 2005) and cleavage of Scc1 by separase at the onset of anaphase (Sumara et al., 2000). Interestingly HP1α and HP1γ have been identified to bind to a kinetochore complex subunit in human

cells: the hMis12 core complex. In the current model HP1 anchors hMis12 at the centromere and enables the formation of the kinetochore scaffold (Obuse et al., 2004). Another protein which is directly associated with hMis12 is ZW10 interacting protein 1 (Zwint-1). Zwint-1 associates with the outer kinetochore during prophase of mitosis and may act as a adapter between the outer kinetochore and the kinetochore core complex (Lin et al., 2006; Obuse et al., 2004). Zwint-1 binds Zeste White 10 (ZW10), that is required for chromosome motility and spindle checkpoint progression (Basto et al., 2000; Chan et al., 2000; Wang et al., 2004a) and also interacts with a centrosomal protein Hec1 (Lin et al., 2006).

Here I report that Odf2 / Cenexin plays an important role during mitosis through the previously identified direct interaction with the Retinoblastoma protein (Rb) (see 3.3). Recent studies suggest that an Odf2 / Cenexin knock down results in chromosome segregation defects mediated by its direct interaction with Plk1 (Soung et al., 2006). However the observed segregation defects may not to be due to the described Odf2 / Cenexin Plk1 interaction. Odf2 / Cenexin knock down cells show a decrease in H3S10ph. H3S10ph in turn is important for chromosome condensation in prophase (Wei et al., 1998) and condensation defects may lead to chromosome segregation failures.

My results show that the knock down of Odf2 / Cenexin results in lagging chromosomes / chromosome segregation defects and to a delay / arrest of cell cycle progression. However this effect is not due to Plk1. Proper chromosome segregation is controlled by coordinated interplay of centrosomes, mitotic spindles, kinetochores, condensing of chromosomes and cohesion of sister chromatids (Cleveland et al., 2003). Odf2 / Cenexin may link centrosome, microtubule attachment, chromosome condensation and separation of chromosomes and so become an important protein for the maintenance of the genetic integrity and prevention of aneuploidy.

Material and Methods

Plasmid Design, siRNA and Transfection

Constructs used are 13.8NC-GFP (Huber et al., 2008), and hCenexin-GFP (pShutttleEGFP_hCenexin; kindly provided by Kyung S.Lee). For cloning expression constructs encoding Zwint1 the coding sequence (Clone ID 5359154; MMM1013-7512658, full length clone by open biosystems in pCMV-Sport6) was used as PCR template. The sequence was amplified using Zwint1_2f (5'-cgaattctgATGGCGGACGCGGAGAAAAAC3-') and Zwint1_2rev primers (5'-GTgcggccgcTgaccggtggAGGAGATTTGGATTTGGCTG-3'). EcoRI vs. BshT1 restriction sites were used for cloning into pdsRed2-N1 (Clontech).

Transfection of plasmid DNA was carried out with Transfectin (BioRad, Munich, Germany) 24 h before fixation or biochemical approaches. *Stealth* siRNA (ODF2MSS207236 and ODF2MSS207237, Invitrogen, Karlsruhe, Germany) was transfected using siLentFect (BioRad, Munich, Germany).

Cell Culture and Synchronization

NIH3T3 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10 % (v/v) fetal bovine serum (FBS), 1000 U/mL penicillin, 1000 µg/mL streptomycin, and 20 mM L-glutamine at 5 % CO_2 and 37°C.

For synchronization at G0 an exponentially growing cell culture were kept in 10 % serum-containing medium for 24 h after they reached confluence. Followed by serum-starvation (0,5 % FBS) for 48 h. For cell cycle arrest at G2 / M transition 3T3 cells were grown until they reached confluence followed by treatment with 400 ng/mL nocodazole for ~ 16 h. For the release from G2 / M arrest the cells were washed twice with normal growth medium and they were allowed to grow until they were analyzed. For G1 / S boundary a subconfluent G0 arrested culture was generated: Approximate 18 h after seeding cells were washed twice with PBS and once with DMEM containing 0,5 % FBS to remove loosely attached, floating cells and residual medium. Cells were incubated in DMEM supplemented with 0,5 % FBS 24 h. For G1/S boundary arrest the medium was changed to DMEM complete containing aphidicolin at 5 µg/mL and incubation for 16 h.

Biochemical Analyses of Protein Levels

Cells were transfected as described above, trypsinized, rinsed with PBS, and counted. Lysates were prepared by adding an equal volume of electrophoresis sample buffer (in 1 x PBS) and heating to 95 °C for 10 min. The proteins of either 10^6 or 10^7 cells were resolved on a 10 % SDS - PAGE and electrophoretic transferred to nitrocellulose membranes (Hybond ECL Nitrocellulose Membrane, GE Healthcare, Munich, Germany). Antibodies against H3S10ph (ab47297), H3K9me3 (ab8898), and Aurora B (ab2254) are commercial provided by abcam (Cambridge, UK). Antibodies against alpha tubulin (Oncogene research products, Merck, Darmstadt, Germany) and H3acetyl (upstate, Millipore, Schwalbach/Ts., Germany) were used.

Immunofluorescence Microscopy

The protocol was adapted as previously described (Huber et al., 2008). Briefly, cells cultured on coverslips were fixed in 3,7 % (w/v) PFA in PBS, pH 7,6 for 20 min. Cells were then permeabilized in 0,5 % (v/v) Triton-X-100 in PBS for 15 min. After blocking with 1 % (w/v) BSA, 0,25 % (v/v) Tween-20 in PBS for 1 h, cells were incubated with the primary antibodies

in blocking solution at 4°C over night. Primary antibodies against alpha tubulin (Oncogene research products, Merck, Darmstadt, Germany), gamma tubulin (GTU88, Sigma-Aldrich, Germany) H3S10ph, H3K9me3, Aurora B (ab47297, ab8898, ab2254, all abcam, Cambridge, UK), and HP1α (Millipore, Schwalbach/Ts., Germany) were used as recommended. Antibodies against Smc3 (1:300; kindly provided by R. Jessberger), Scc2 (1:200; kindly gift by J.M. Peters), and human CREST serum (a kind gift of A. Kromminga) were used in the given concentrations. After washing three times for 10 min with PBS the specimens were incubated with the appropriate secondary MFP conjugated antibodies (MoBiTec, Göttingen, Germany) and DAPI (1 µg/mL) in blocking solution for 1 h at RT. After washing three times 10 min with PBS cells were mounted in VectaShield (Linaris, Wertheim, Germany).

For analysis of mitotic cells, cells were synchronized as described above and collected by mitotic shake-off. Cells were washed twice with complete growth medium without nocodazole and incubated for 1 hour at 37°C and 5 % CO_2 in complete DMEM. Mitotic cells were spun onto glass slides for 5 min at 1400 rpm and immunostaining was carried out as described above.

FACS

For cell cycle analyses by FACS assay cells were synchronized as described previously. For each analysis 10^6 cells were harvested and fixed in EtOH. Cells were washed twice in PBS followed by RNase digestion for 30 min at 37°C. DNA was stained with propidium iodide.

Results

Knock down of Odf2 by RNAi

For functional analysis of Odf2 / Cenexin siRNAs were transfected. Two different Odf2 / Cenexin siRNAs (*stealth* siRNA, Invitrogen, MSS207236 and MSS207237) were tested and the reduction of the protein was determined at different time points.

The reduction of the cellular protein level was analyzed by western blot using polyclonal rabbit anti Odf2 antibodies (Figure 1a). The reduction level was calculated by integration of the pixel density of blots using ImageJ 64 software. Expression level of control cells is set to 100 % (Figure 1b).

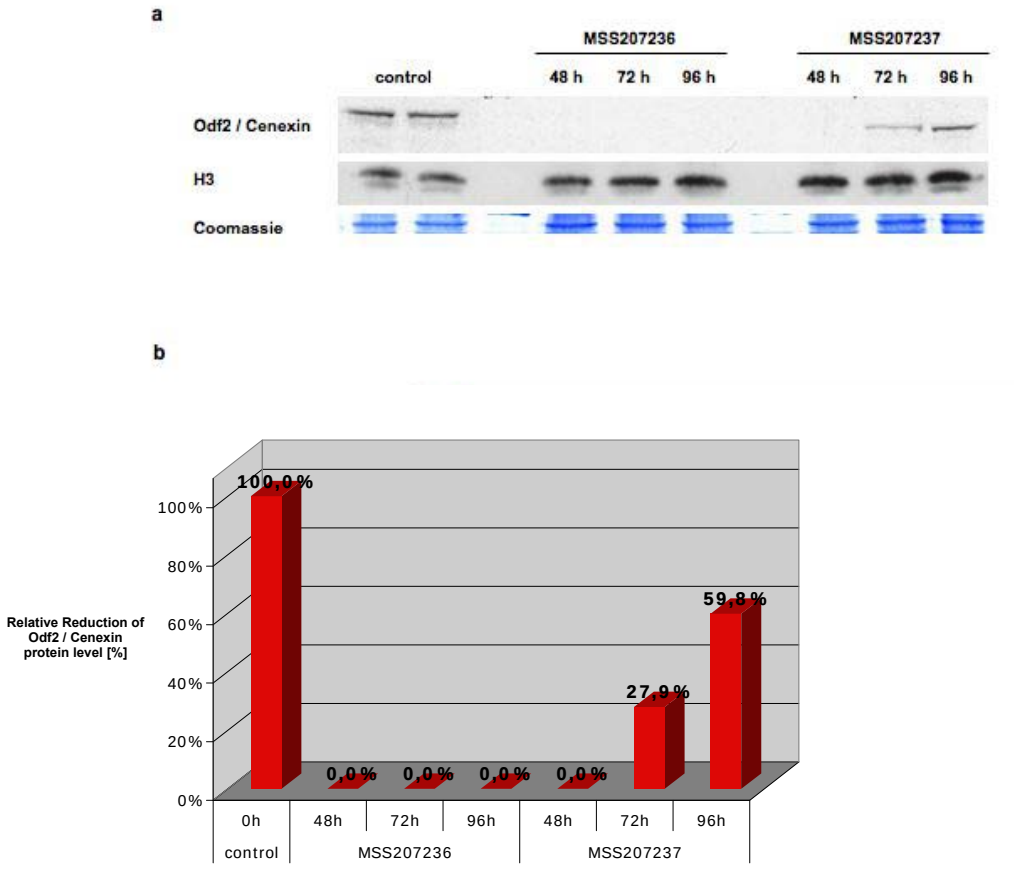

Figure 1: Odf2 / Cenexin RNAi (a) Immunoblotting analyses of Odf2 / Cenexin depletion by siRNA transfection. Reduction of the Odf2 / Cenexin protein level was determined 48 h, 72 h, and 96 h after siRNA transfection. (b) Reduction was calculated using ImageJ64 software. The diagram compares control cells with RNAi cells plotted for different time points after transfection.

Reduction below the detection limit could be achieved using RNAi. No detectable expression of Odf2 / Cenexin was determined 48 h after transfection of both used siRNAs (MSS207236 or MSS207237). Weak recovery of Odf2 / Cenexin protein level could be detected 72 h after transfection with MSS207237 siRNA. After 96 h 60 % of the wildtype level was recovered. MSS207236 mediated a 100 % reduction of the protein. No Odf2 / Cenexin could be detected in transfected cells after 48 h, 72 h, or 96 h. For further experiments siRNA MSS207236 was used and effects were investigated 48 h after transfection.

The centrosomal protein Odf2 / Cenexin influences posttranslational histone modifications

In the previous part of this thesis I have demonstrated the direct interaction of Odf2 / Cenexin with the retinoblastoma protein (Rb, see 3.3). Rb is known to interact with a variety of transcription factors and chromatin modifying enzymes, which contribute to both heterochromatin and gene regulation (Isaac et al., 2006; McCabe et al., 2005; Siddiqui et al., 2007). I was able to exclude effects on E2F promoters by a reportergene assay described in 3.3 and therefore any influence on E2Fs and their transactivation function caused by this

interaction. I addressed the question if the interaction may effects chromatin by modifications of histones. I used RNAi mediated knock down of Odf2 / Cenexin to determine the levels of posttranslational histone modifications through indirect immunofluorescence and western blotting.

To examine the effect of Odf2 / Cenexin RNAi cell lysates of untreated and siRNA transfected cells were prepared and the cellular levels of diverse proteins were examined by SDS – PAGE and western blot (Figure 2). Alpha tubulin was detected to verify equal loading of cells / proteins.

RNAi mediated depletion of Odf2 / Cenexin cells showed a decrease of H3S10ph. Aurora B, which has been described to phosphorylate H3S10 (Crosio et al., 2002; Giet and Glover, 2001), showed unaltered protein level by immunoblotting. Interestingly there were two bands detected by the antibody. The shifted one may be the phosphorylated form of the kinase. The Aurora B level is only very weakly if it all decreased after Odf2 / Cenexin RNAi. The H3K9me3 is not affected. The expected increase caused by the decreasing phosphorylation on S10 is not noticeable. And the examined level of acetylated H3 was not affected.

Indirect immunofluorescence allowed counting H3S10 phosphorylated NIH3T3 cells (Figure 3) and distinguish mitotic and transcriptional phosphorylation on histone 3 serine 10 by morphology. In control cells 55 % of an exponential growing culture were not marked by phosphorylation on H3S10 whereas in 45 % of cells the histone modification was detectable (total number of cells counted: 897). In Odf2 / Cenexin RNAi cells only 12 % were marked by phosphorylation at H3S10 (total 943 cells counted). This effect was uncoupled to the confluence of the culture stained by antibodies against H3S10ph. In both samples mitotic and transcriptional S10 phosphorylation could be observed.

The decrease of S10 phosphorylation at H3 has not influenced the H3K9me3 what is functionally linked to phosphorylation (Fischle et al., 2005). In control as well as in RNAi cells an equal amount of trimethylated lysine 9 was detected by western blot analyses (Figure 2) and indirect immunofluorescence staining did not show any differents (Figure 4). H3K9me3 has been detected associated with the heterochromatic regions in control and RNAi cells. No increase determined by blotting or a stronger signal measured by confocal microscopy could be detected. The H3K9me3 mark was also examined in Odf2 / Cenexin overexpressing cells through by transfection of p13.8NC-gfp using immunofluorescence staining. The majority of these cells showed a staining in the nucleus and a reduced association with the heterochromatin. These euchromatic pattern were found in control cells prevalent beside the heterochromatic association.

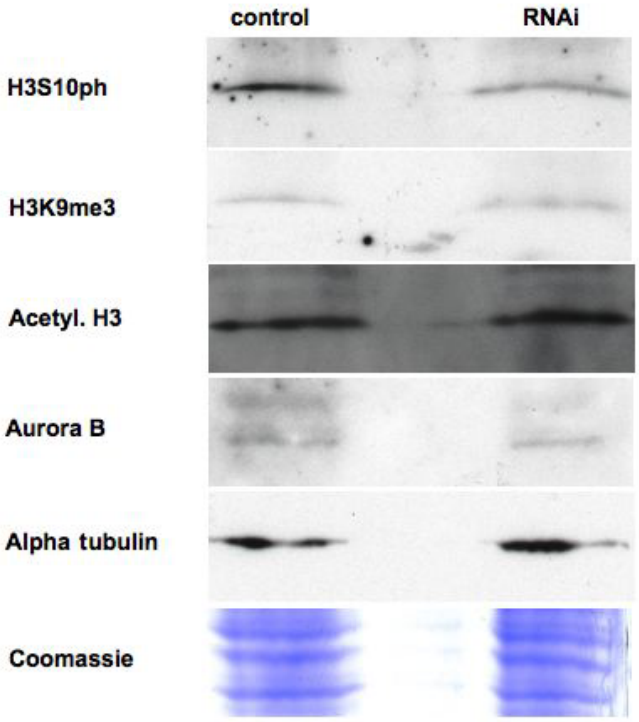

Figure 2: Determination of protein level after Odf2 / Cenexin RNAi. Comparative equal amounts of untreated and RNAi cells were loaded (see loading control staining by anti alpha tubulin antibodies and coomassie staining). While H3K9me3 and H3 acetylation is not effected, phosphorylation of H3 is decreased after RNAi. The Aurora B level is weak decreased rather not affected.

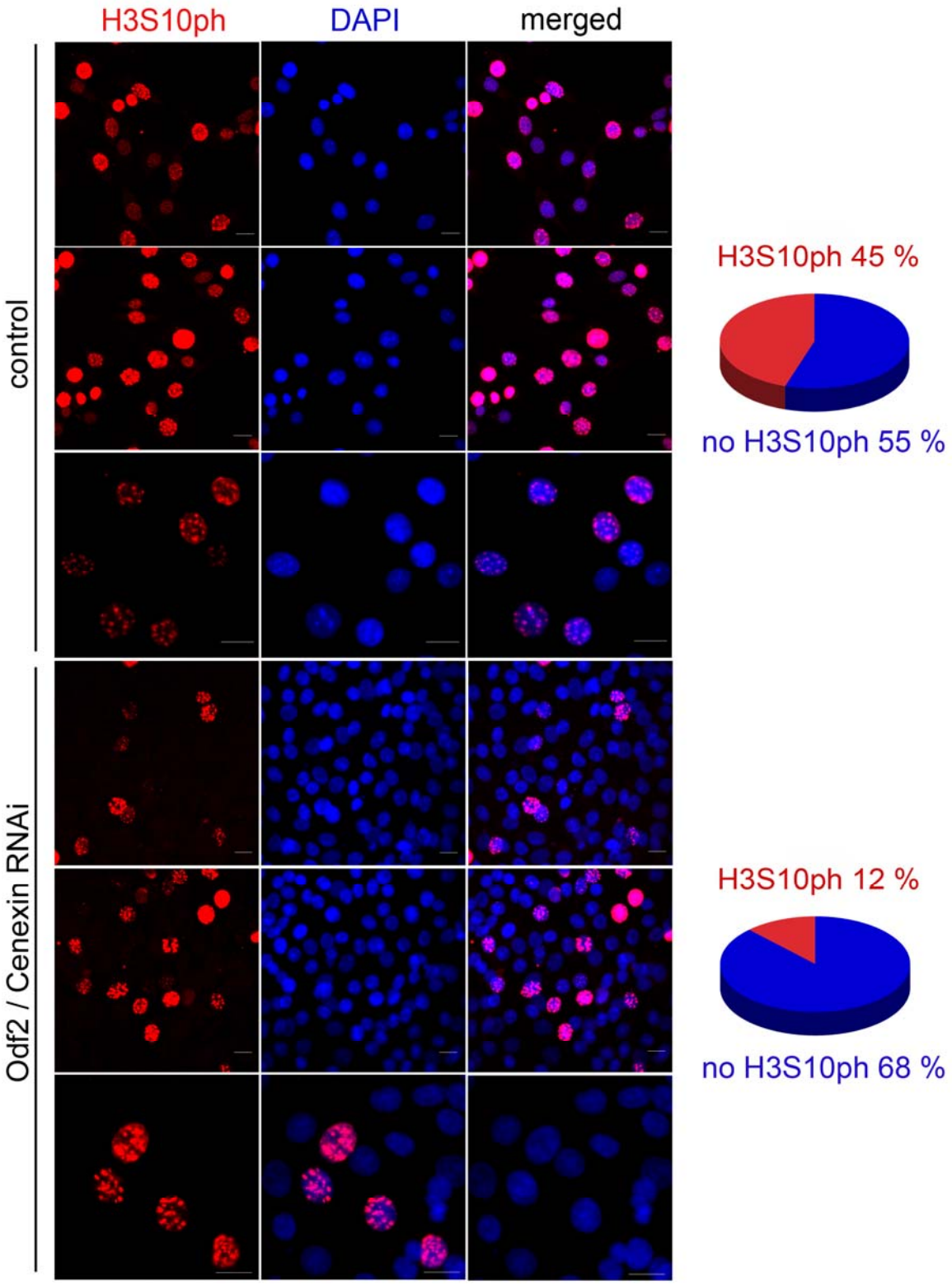

Figure 3: Odf2 / Cenexin RNAi leads to a decrease of H3S10ph. Only 12 % of cells show H3S10ph after Odf2 / Cenexin RNAi while 45 % of control cells show this posttranslational histone modification. RNAi does not effect the heterochromatic associated localization. (Bars 5 µm).

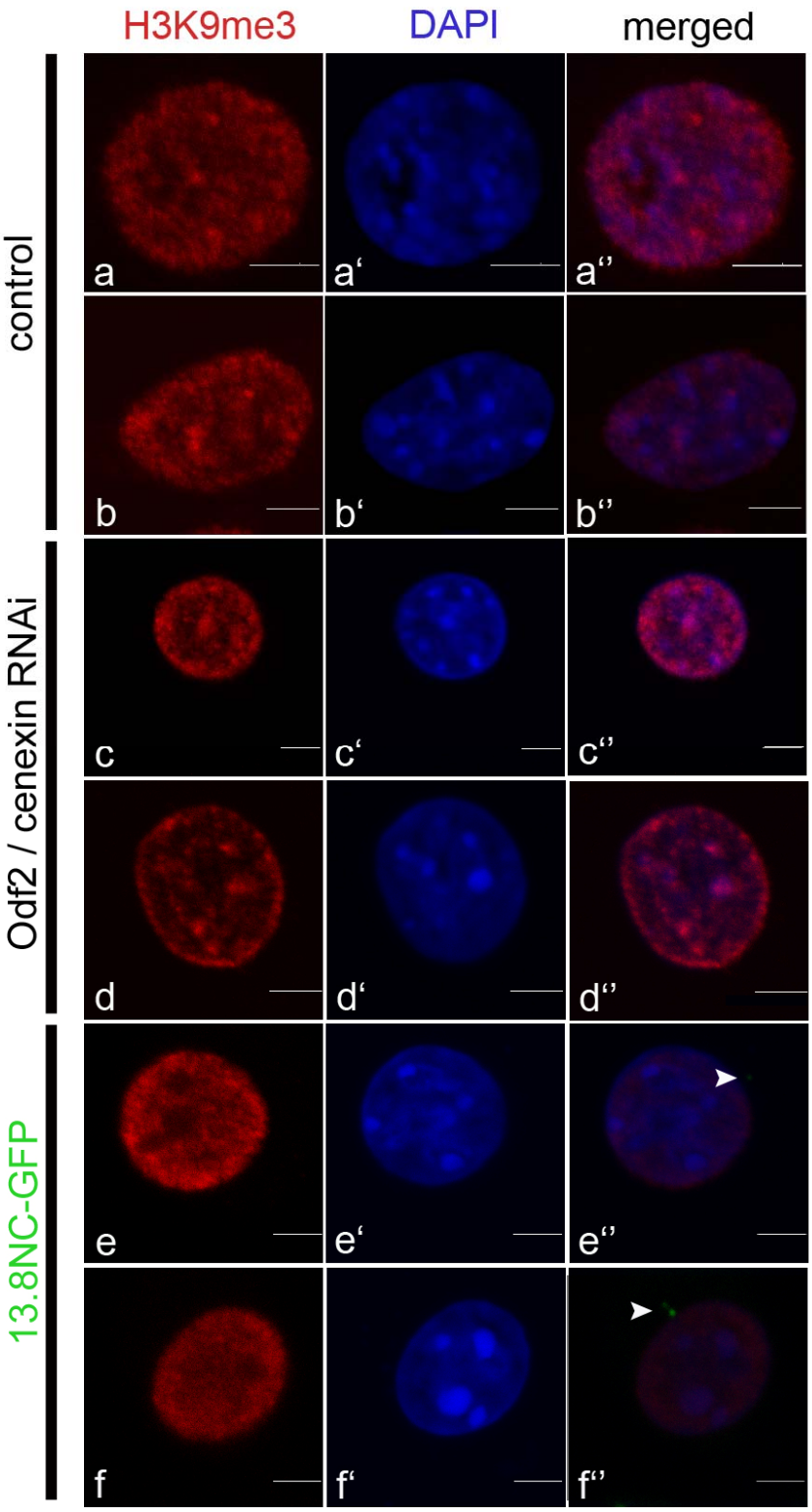

Figure 4: H3K9me3 in control (a – b), RNAi (c – d), and overexpressing cells (e – f). The H3K9me3 mark is not effected by RNAi or overexpression of Odf2 / Cenexin. In overexpressing cells, the centrosomal localization of the GFP – Odf2 fusion protein is marked by arrowheads; prevalent the modification is not associated with heterochromatic regions. (Bar 5 µm).

At last the Aurora B expression pattern was analyzed. The immunoblotting analyses has shown a very weak rather no decrease of Aurora B levels. The antibody detected two bands. The upper one may represent the phosphorylated, active form of the kinase (Figure 2). Indirect immunostaining revealed no delocalization or differences in expression levels in RNAi cells (Figure 5). All cells showed the expected distribution during mitosis.

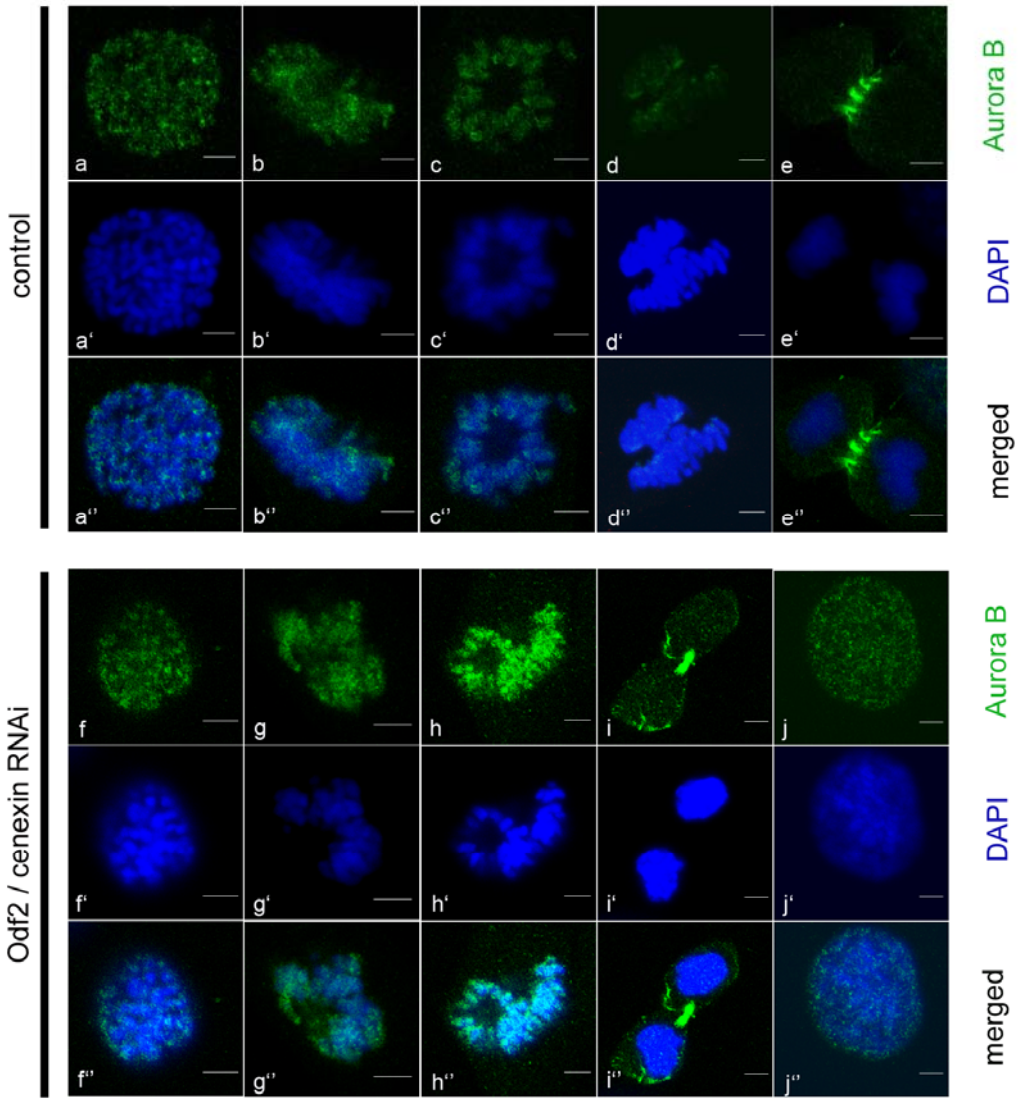

Figure 5: Aurora B staining in mitotic cells [5 µm]. No differences in the localization are detectable by microscopy analyses of control (a – e) and RNAi cells (f – i). In analyzed mitotic cells problems with chromosome segregation were often detected after Odf2 / Cenexin RNAi. Distribution in interphase cells after Odf2 / Cenexin RNAi is show in j – j''and did not differ to those observed in control cells.

Is the decrease of H3S10 phosphorylation a mitotic or transcriptional effect?

To determine if the decreased level of H3S10ph is a mitotic or transcriptional effect I synchronized cells at the G1/S boundary and the G2/M transition. The synchronization was verified by FACS analyses (see figure 6a). Cells were arrested at the G1/S boundary and released for 3 h. The cells synchronized at G2/M transition were released for 30 min. Both harvest by trypsinisation. After the release and the transition to S and M phase respectively cell lysates were prepared and analyzed by immunoblotting (figure 6b). The effect of Odf2 / Cenexin RNAi was determined for both samples. The analyzis of the H3S10ph mark showed a much stronger decrease of H3S10ph in mitotic cells compared to S phase cells. Odf2 / Cenexin thus seems to positively regulate mitotic H3S10ph.

Therefore I focused further investigations on mitotic cells to determine Odf2 / Cenexins functional role.

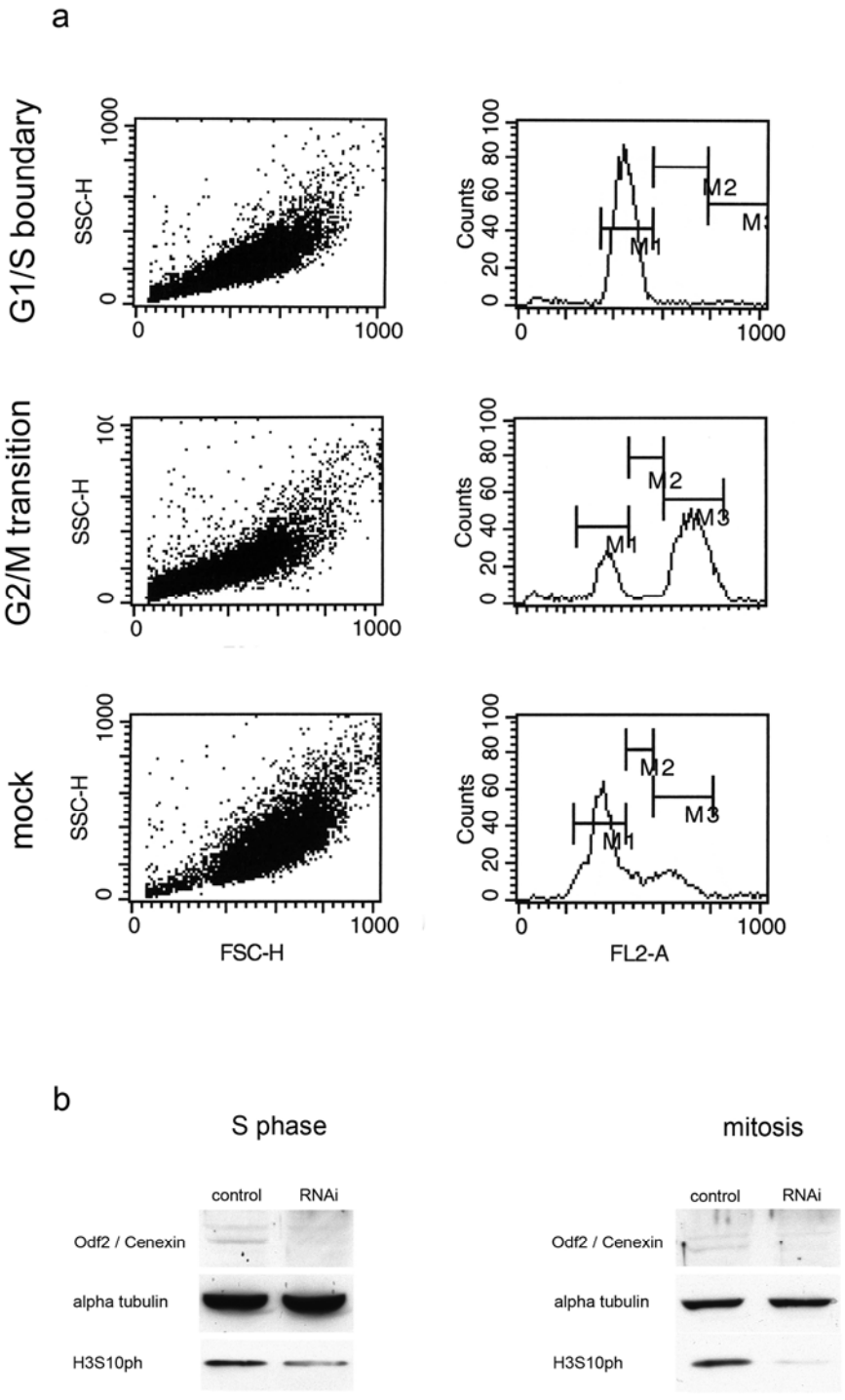

Figure 6: Cells were arrested at the G1/S boundary and G2/M transition and released. The successfully arrest was verified by FACS analysis (a). Nearly the complete cell population was arrested in G1/S boundary shown by the large peak M1. Most of the cells were arrested at G2/M transition. Cells in mitosis are represented by peak M3, cells in G2 by M1. The mock control showed the features of an exponentially growing culture displayed by no clearly determined peaks. b: Analysis of the cell lysates showed an stronger decrease of the H3S10ph mark in mitotic cells.

Chromosome Condensation or Sister Chromatid Cohesion – two headliners concerning chromosome segregation defects during mitosis checked after Odf2 / Cenexin RNAi

After the finding that Odf2 / Cenexin is linked to mitotic H3S10ph as well as to the retinoblastoma protein I examined proteins which play important roles in mitotic events. These proteins had to localize to kinetochores or centromeric regions and function in cell cycle events through establishment of mitotic spindle attachment or sister chromatid cohesin. The described association of Plk1 with Odf2 / Cenexin could link the observed chromosome segregation defects to sister chromatid cohesion.

Odf2 / Cenexin knock down causes chromosome misalignments. Therefore Smc3, a protein which is necessary to establish sister chromatid cohesion was analyzed in mitotic and interphase cells after Odf2 / Cenexin RNAi (Figure 7). In control cells Smc3 localizes to the nucleus and the antibody gave predominant nuclear staining in all investigated interphase cells. Heterochromatic regions were not stronger stained (compare HP1alpha staining and DAPI) and the nucleolar regions are mostly spared. It is known that the protein is dislocalized during anaphase and dissociates from chromatin, allowing sister chromatids to separate. Smc3 could not be detected on mitotic chromosomes (Figure 7 c – e). In RNAi cells the same pattern of Smc3 localization was observed (Figure 8). Odf2 / Cenexin RNAi did not effect the localization of the cohesin component Smc3.

Next Scc2 another component of the cohesin complex was analyzed by IF (Figure 9). In untreated control cells Scc2 was detected homogenously distributed in the nucleus, because no pre – extraction prior fixation was carried out. A major fraction of Scc2 displays cytoplasmic localization. Heterochromatic regions are not preferentially stained (see DAPI). However in Odf2 / Cenexin RNAi cells Scc2 associated strongly with the nucleoli but the nuclear allover staining could be observed like in control cells.

At last I had a look on centromeres during mitosis by staining with hCREST positive serum (Figure 10). In control cells the MTs of the mitotic spindle properly attach to the kinetochore complex identified by the hCREST and alpha tubulin staining. Separation of sister chromatids to the spindle poles occurs as cells progress through the cell cycle. In contrast RNAi cells show no association of hCREST with spindle MTs, misaligned and missegregated chromosomes.

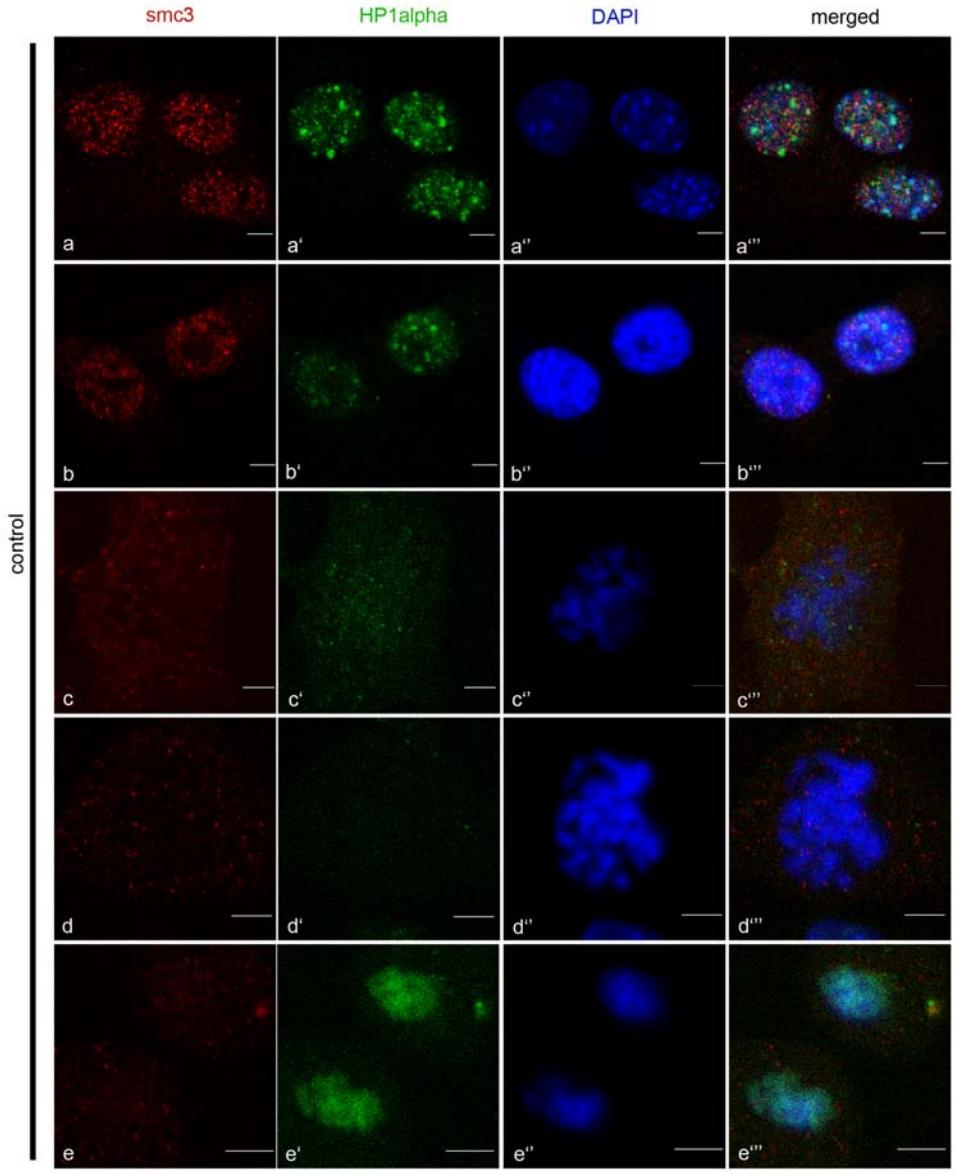

Figure 7: Intracellular localization of Smc3 in control cells (bar 5 µm). In interphase cells Smc3 localized predominantly to the nucleus, heterochromatic regions are not preferentially stained (a + b). In mitotic cells Smc3 is removed from chromatin (c - e).

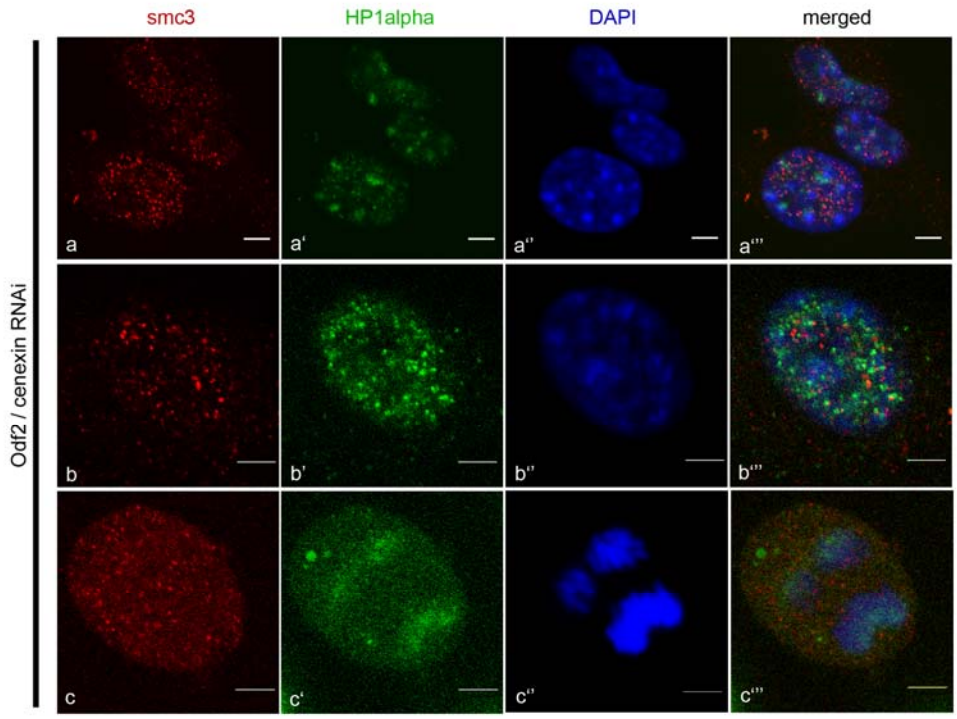

Figure 8: Staining of Smc3 in RNAi cells. The predominant nuclear staining is still shown (a + b). Cells often showed aberrant chromosome segregation (c).

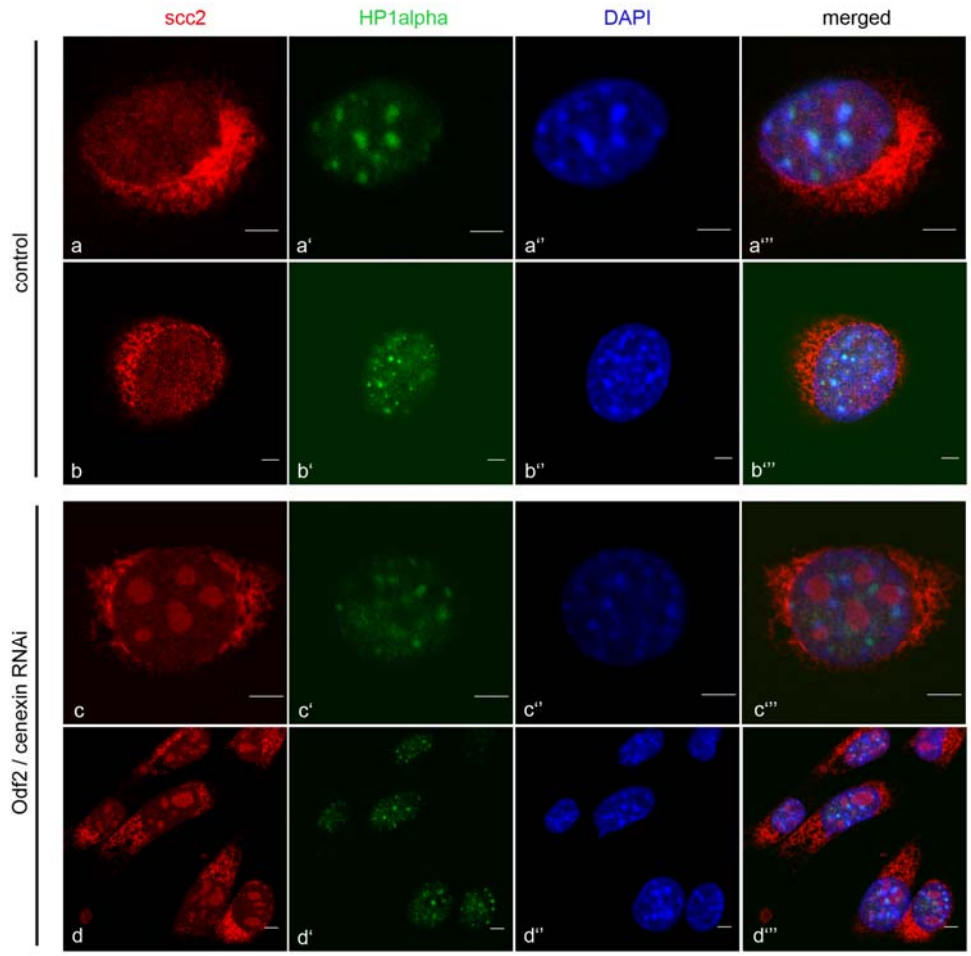

Figure 9: Localization of Scc2 and Hp1alpha in control (a + b) and RNAi cells (c + d). Scc2 was detected in the nucleus in a predominant uniform pattern. In RNAi cells a stronger association of Scc2 with the nucleoli was recognized.

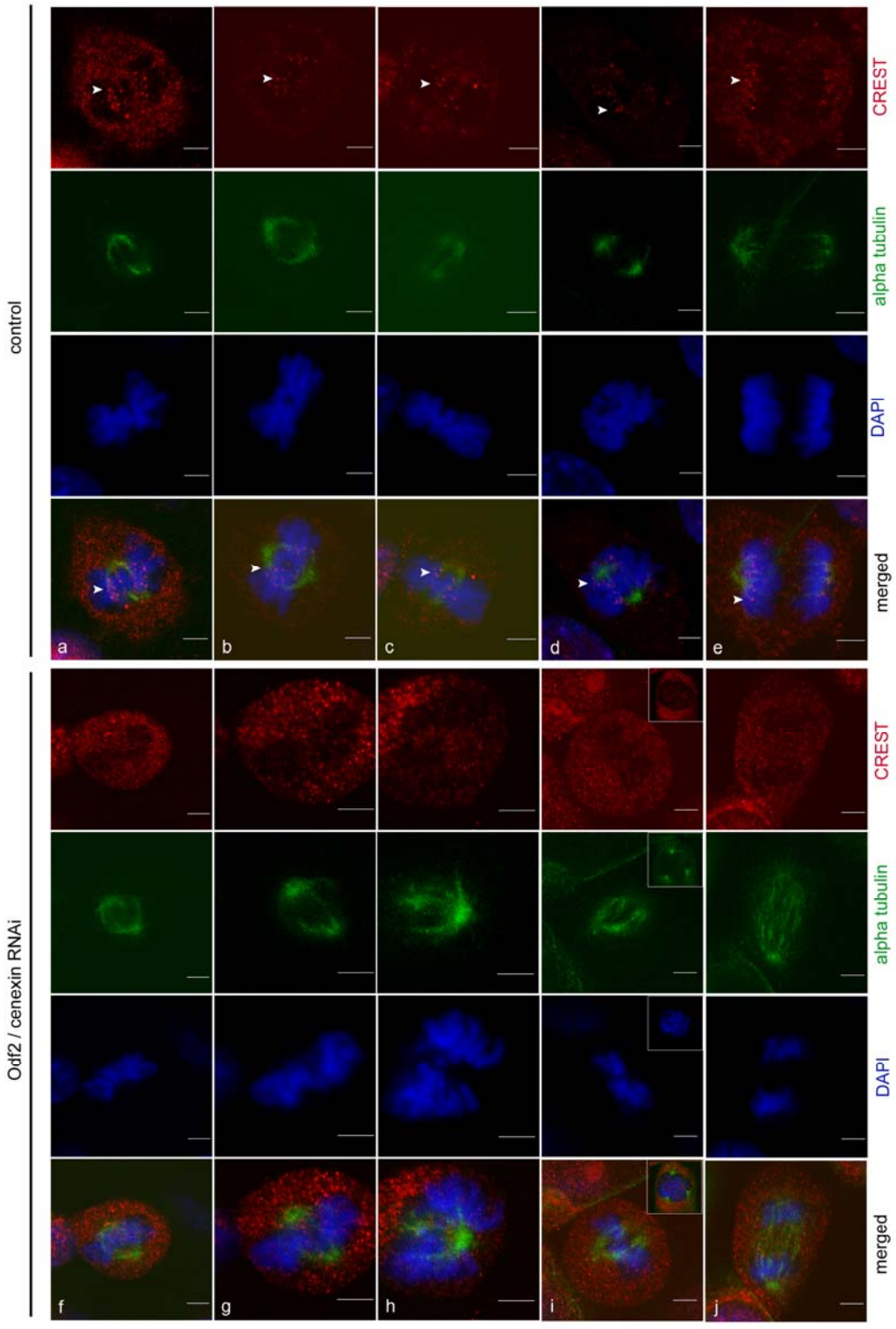

Figure 10: Detection of centromeres after RNAi mediated deplation of Odf2 / Cenexin by hCREST sera. In control cells the centromeric regions are localized associated with the ends of the spindle microtubules (arrowheads, a – e). In RNAi cells the centromeric regions are often no longer detectably associated with the microtubules of the mitotic spindle. Missegregated chromosomes and spindle defects are observed (e.g. i – and inset in i)

Interaction of Odf2 / Cenexin with the kinetochore protein Zwint - 1

The observation of mitotic defects in Odf2 / Cenexin RNAi cells and the notice that defects through depletion of Odf2 / Cenexin actually did not result in centrosomal aberrations, I had a closer look at Zeste white interacting protein 1 (Zwint - 1). Zwint - 1 has been identified previously as a putative interaction partner of Odf2 / Cenexin by a yeast two hybrid screen and is known as a kinetochore associated protein.

First I looked at the intracellular localization of Zwint-1. It has been described that Zwint-1 is localized at the kinetochores during mitosis, but recently published data did not include localization studies of interphase cells (Lin et al., 2006). My results show that overexpressed dsRed – Zwint - 1 fusion protein co – localized with the gamma tubulin at the centrosomal region in interphase cells (Figure 11). The magnification shows that each gamma tubulin stained dot is colocalizes with up to two Zwint - 1 speckles (i - iii).

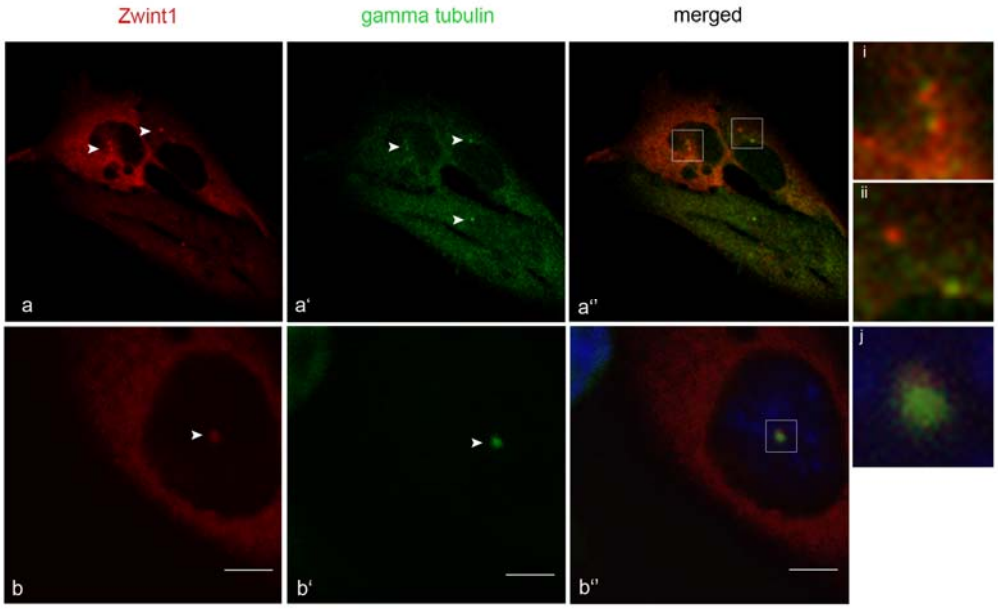

Figure 11: The dsRed Zwint - 1 fusion protein localizes in the centrosomal region stained by anti gamma tubulin antibodies. i: Magnification of the association of Zwint - 1 with gamma tubulin of cells shown in a. ii and j show association between one gamma tubulin and one Zwint - 1 dot; j is the magnification of b (bar 5 µm).

Discussion

The Histone H3 serine 10 phospho mark is considered a marker for mitotis in the eukaryotic cell cycle (Prigent and Dimitrov, 2003). Recent studies support the hypothesis that this modification is involved in a pathway of higher order chromatin folding or / and unfolding. It is discussed that phosphorylation of H3 may cause a local and transient decondensation of chromatin by reducing the interaction between H3 amino termini and DNA. This may be the first step to facilitate the binding of other trans – acting chromatin condensation factors like SMC proteins (Wei et al., 1998). SMC proteins (proteins with a 'structural maintenance of chromosomes' ATPase subunits) are involved in forming multi – subunit protein complexes involved in condensation of chromosomes and cohesion of sister chromatids.

Here I report a decrease of H3S10ph caused by Odf2 / Cenexin RNAi. Effects of the H3K9me3 / H3S10ph switch were excluded by indirect immunofluorescence and immunoblotting analyses. Only the H3S10ph was affected and no influences on lysine 9 trimethylation could be detected. I was able to narrow down the effect mostly on mitotic events by examination of the H3S10ph level of mitotic cells in comparison with G2 cells. It was possible to exclude upstream defects through investigation Aurora B, the kinase that is described to be required for H3S10 phosphorylation. Neither the level of Aurora B nor its intracellular localization was affected by Odf2 / Cenexin RNAi.

Aurora B has not been affected by Odf2 / Cenexin RNAi but nevertheless an effect on H3S10ph could be observed. Odf2 / Cenexin RNAi cells often display chromosome segregation defects also observed by Soung *et al*.. Recent studies link the segregation defects to Plk1 (Soung et al., 2006). Plk1 is involved in correct separation of sister chromatids by phosphorylation of the cohesin protector Shugoshin (SgoI). Unphophorylated SgoI blocks the phosphorylation of the cohesin subunit Scc1 and protects Scc1 from degradation by Separase and therefore prevents premature loss of centromeric cohesin. Once SgoI becomes phosphorylated centromeric cohesin can be removed by Separase activity (Hauf et al., 2005; Yamagishi et al., 2008). Plk1 (not Aurora B) is also implicated to be required for dissociation of cohesion from chromosome arms in early mitosis by triggering phosphorylation of Scc1 and SA2 (Hauf et al., 2005).

I tested the localization of cohesin components Scc2 and Smc3 by indirect immunofluorescence. Odf2 / Cenexin siRNA treated cells do not show altered localization of Scc2 and Smc3. Therefore a link between the described segregation defects and sister chromatid cohesion could not be established. Scc2 remains chromosome bound from telophase to prophase. Moreover the localization of HP1α is not effected. The removal of centromeric cohesin should eject HP1 from chromatin (Pidoux and Allshire, 2004; Yamagishi

et al., 2008). Control as well as RNAi cells show HP1α dislocation during mitosis and reassociation in telophase.

However segregation failures can also be caused by wrong chromosome condensation and the fact that I was able to describe a physically interaction of Odf2 / Cenexin and the retinoblastoma protein could be an alternative explanation of the observed effects. In *Drosophila* a physically interaction between RBF1 (*Dm* homologous of Rb) and dCAP-D3 has been described (Longworth et al., 2008). RBF1 promotes the association of dCAP-D3 with chromatin meaning that Rb is required for normal chromosome condensation. CAP-D3 is a subunit of the condensin II multi – protein complex involved in initiation of chromosome condensation in prophase. The complex is formed by Smc2 and Smc4, a kleisin subunit and two HEAT repeat proteins. CAP-D3, CAP-H2, and CAP-G2 are associated with the complex and are targets of e.g. cdk1 phosphorylation (Aono et al., 2002; Nasmyth and Haering, 2005; Onn et al., 2007).

It has been shown that Aurora B depletion has little if any effect on the ability of condensin to bind to chromosomes (Sumara et al., 2000) but H3S10ph has been proven to play a crucial role in chromosome condensation in prophase during mitosis (Prigent and Dimitrov, 2003; Wei et al., 1998). Therefore the uneffected Aurora B level as well as the H3S10ph decrease after Odf2 / Cenexin RNAi fit the theory. Further investigation like the role of the condensin complex components will be necessary to support this model. The conclusion from my results fit the working model established by Longworth *et al.* who reported that RBF1 overexpression leads to an increased dCAP-D3 association with DNA and result in hypercondensation whereas a loss of RBF1 expression causes hypocondensation. The demonstrated loss of Rb expression through Odf2 / Cenexin RNAi and the Rb interaction with Odf2 / Cenexin (see 3.3) possibly effects CAP-D3. Perhaps this putative association leads to a decrease of CAP-D3 which may result in hypocondensation. Additionally, the decrease of the H3S10ph mark also causes hypocondensation that leads to inefficient resolution of sister chromatids, anaphase defects such as lagging chromosomes and chromosome bridges (Lam et al., 2006; Savvidou et al., 2005) and finally cause aneuploidy and cancer.

The association of Odf2 / Cenexin and Zwint - 1 a kinetochore protein could be shown. Recently I was able to determine a centrosomal co - localization of both proteins. If the interaction will be confirmed, I would expect problems of spindle microtubule attachment to centromeres / kinetochores after Odf2 / Cenexin knock down.

In control cells the centromere regions are well defined structures. These structures can be stained with hCREST positive sera and an association with the plus ends of the spindle microtubules can be visualized. This strong association with the microtubule tips seems to be lost in RNAi cells. The centromeres also seem to be arranged in a dispersed manner in

metaphase. However the chromatids are pulled to the spindle poles and seem to be equally distributed.

This findings can be an interesting hint in terms of the Odf2 / Cenexin localization during mitosis. Odf2 / Cenexin is a centriolar protein localized to the appendages of the mother centriole (Lange and Gull, 1995; Nakagawa et al., 2001). It has been described that the appendage proteins are not visible in mitosis until cytokinesis (Azimzadeh and Bornens, 2007). Laser scanning microscopy of endogenous Odf2 / Cenexin by indirect immunostaining or overexpression of GFP - fusion constructs corroborate the theory. In all mitotic cells observed I was not able to detect Odf2 / Cenexin associated with the centrosome. In agreement with Azimadeh *et al.* and what they have described regarding centriole appendage proteins Odf2 / Cenexin seems to be dislocated. So far, I could not narrow it down to a mitotic stage. Therefore it could be possible that Odf2 / Cenexin is dislocated from the centriole and associates with the kinetochore during mitosis recruited by the kinetochore protein Zwint - 1. This has to be proven in future research.

I postulate that the chromosome segregation effects triggered by Odf2 / Cenexin RNAi are not linked to the Plk1 mediated “sister chromatid segregation pathway” described above. Future work will have to demonstrate that the observed chromosome segregation defects are an upstream effect and a consequence of failure in chromosome condensation. Furthermore, there might be an association of Odf2 / Cenexin and Zwint - 1 and therefore a link between centrosome and kinetochore attachment. However it will need further analyzis to investigate the functional link of both proteins.

In summary I assigned the observed chromosome segregation defects after Odf2 / Cenexin RNAi to chromosome condensation defects rather than to defects in sister chromatide cohesion. Odf2 / Cenexin thus becomes a crucial protein that prevents cells from aneuploidy and finally cancer through maintaining the genetic integrity.

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4. Discussion

In this study a somatic splice variant of Odf2 / Cenexin has been isolated and characterized. I was able to show that Odf2 and Cenexin transcripts are the products of differential splicing of a single gene (Huber and Hoyer-Fender, 2007). The described variant 13.8NC posses an 42 amino acid N – terminal extension encoded by a novel exon that not has been described before in the *Odf2* gene sequence (Hoyer-Fender et al., 2003). In this exon an alternative translation start codon could be mapped. This translation start was exclusively linked to the gene product Cenexin. Odf2 isoforms that first have been isolated from testes do not contain the 42 amino acid insertion. Analyses of transcripts determined an association of *cenexin* with somatic cells whereas the bulk of *odf2* is associated with testicular germ cells. These associations are not completely excluded to the cell type. *Cenexin* transcripts could also be detected by RT-PCR analyses of testicular tissue. However, it has to be kept in mind, that in testis the germ cells are surrounded by somatic cells (e.g. epithelial cells, Sertoli cells and Leydig cells) and that cDNA used for analyses was transcribed from total tissues. To conclude, Cenexin has been found to be ubiquitously and equally expressed in all tissues analyzed, whereas Odf2 expression is up regulated in testes and correlates with ongoing spermatogenesis and sperm tail formation.

Next I was interested to address whether the N – terminal extension which seems to be linked to somatic cells influences the intracellular localization of the protein and potential protein interactions. The testis variant Odf2 was functionally linked to the stabilization of the mammalian sperm tail through formation of the outer dense fibers. Furthermore it has been identified as a centrosomal scaffold protein (Baltz et al., 1990; Hoyer-Fender et al., 2003; Hoyer-Fender et al., 1998; Nakagawa et al., 2001). Early publications also described Odf2 within the centrosome as a fiber forming and stabilizing protein localized to the pericentriolar matrix and associated with the appendages of the mother centriole (Nakagawa et al., 2001). Even though Odf2 has been depicted as a self – interacting microtubules associated protein with a cell cycle dependent expression its cellular more precisely centrosomal function is still unclear (Donkor et al., 2004; Lange and Gull, 1995). I wanted to elucidate if the additional 42 amino acid stretch of the Odf2 / Cenexin protein predominantly expressed in somatic cells undertakes special functions to target the protein primarily to the centrosome in somatic cells. I was able to show that the 42 amino acid stretch is necessary but not sufficient for centrosomal targeting and that a great part of the C – terminal coiled coli region also seems to be important for centrosomal localization (Huber et al., 2008). These findings were

confirmed by Soung *et al.,* who characterized the C-terminal part of the protein (Soung et al., 2006).

Our expression analyses displayed a strong association of this 42 amino acid stretch containing constructs with acetylated microtubules and the primary cilia. Odf2 has been described to be necessary for primary cilia formation and the formation of the distal and subdistal appendages of the centrioles (Ishikawa et al., 2005). However I postulate that Odf2 is not involved in ciliogenesis through initiation of cilia formation. I suggest it acts as a stabilizing protein through its association with acetylated and thereby stabilizing microtubules. I was not able to manipulate cilia formation by Odf2 / Cenexin RNAi (data not shown). These cells went normaly through ciliogenesis. These results are corresponding with findings of another research group, that tried to inhibit primary cilia formation by Odf2 / Cenexin RNAi (personal communication, Francesca Fabretti, University Hospital of Cologne, Cologne, Germany). Another hint of its stabilizing function is derived from analyses of an Odf2 homolog in freshwater planaria. The motility of this worm relies on a ventral multiciliated epithelium. In such epithelia, cells form a large number of basal bodies that must be properly orientated relative to each other and the body axis to allow a synchronous ciliary beating. Depletion of SmedOdf2, the planaria homolog of the vertebrate Odf2, induces a sidewinder phenotype (Reddien et al., 2005) but to date it is unclear if the phenotype is caused by depletion of cilia or a stabilization defect. However, I think the finding that basal bodies mostly are proper positioned could be a hint of correct cilia development. This confirms the hypothesis that Odf2 / Cenexin has stabilization function in ciliogenesis.

For further functional analyses of Odf2 / Cenexin the amino acid sequence was mapped and screened for putative interaction motifs. A so - called LxCxE peptide motif was found. LxCxE peptide motifs are described to mediate interactions between many cellular proteins and the retinoblastoma protein an important cell cycle regulator over the LxCxE binding cleft of Rb. Using biochemical approaches I was able to narrow down the physical interaction between both proteins to the peptide motif and the LxCxE binding cleft on the surface of Rb.

Once more the interaction sheds light on Odf2 / Cenexin as an important protein in cell cycle progression. Its function in cell cycle related processes first has been determined by its interaction with Plk1. It has been described that Odf2 / Cenexin recruits Plk1 over its C – terminal extension to the centrosome and recruitment failures of Plk1 may results in chromosome segregation defects (Soung et al., 2006). Using Odf2 / Cenexin RNAi I investigated effects on cell cycle progression in respect to the Rb interaction. I was able to exclude an influence of Odf2 / Cenexin on E2F transcription factors and their transactivation function through reporter gene assays using a luciferase system. However although E2F is

the best-known Rb – associated protein, there is very clear evidence that Rb has additional targets and functions in multiple pathways.

In *Drosophila* a physically interaction between RBF1 (*Drosophila melanogaster* homologous of Rb) and dCAP-D3 has been described (Longworth et al., 2008). Therefore RBF1 (Rb) could be linked to chromosome condensation at the onset of mitosis. Longworth *et al.* were able to demonstrate an association of dCAP-D3 with chromatin. They postulate Rb is required for normal chromosome condensation. CAP-D3 is a subunit of the condensin II multi – protein complex involved in initiation of chromosome condensation in prophase. The complex is built up from two members of the structural maintenance of chromosomes family Smc2 and Smc4, a kleisin subunit and two HEAT repeat containing proteins. CAP-D3, CAP-H2, and CAP-G2 are associated proteins involved in the formation of the condensing II complex and are targets of phosphorylation e.g. by cdk1 (Aono et al., 2002; Nasmyth and Haering, 2005; Onn et al., 2007).

It has been shown that Aurora B depletion had little if any effect on the ability of condensin to bind to chromosomes (Sumara et al., 2000) but the H3S10ph mark has been proven to play a crucial role in chromosome condensation in prophase during mitosis (Prigent and Dimitrov, 2003; Wei et al., 1998). By performing Odf2 / Cenexin RNAi I was able to show a decrease of H3S10ph in Odf2 / Cenexin depleted cells whereas intracellular localization of Aurora B and its protein levels determined by western blot analyses were not effected.

Furthermore I observed segregation defects of chromosomes in RNAi cells. These defects have already been described by Soung *et al.* and are linked to the Plk1 interaction (Soung et al., 2006). These segregation defects may not be caused by the Plk1 interaction but a consequence of the H3S10ph decrease because segregation defects can also be triggered by wrong chromosome condensation.

If the recruitment of Plk1 to the centrosome is mediated by Odf2 / Cenexin the reason of the defects could this way be linked to sister chromatide cohesion. However, I was able to show that the components of the cohesin complex were not effect by Odf2 / Cenexin RNAi. Vertebrate cells remove the majority of cohesin from chromosome arms during prophase without Scc1 cleavage (Waizenegger et al., 2000). This “prophase – pathway” depends at least in part on SA2 phosphorylation, which appears to be mediated by Plk1 (Sumara et al., 2002). At centromeres a small amount of cohesin is protected from the “prophase – pathway” by the Shugoshin protein SgoI (Salic et al., 2004). Cohesin complexes that are still associated with chromosomes in metaphase are then cleaved by separase (Waizenegger et al., 2000). Unphosphorylated SgoI blocks the phosphorylation of the cohesin subunit Scc1 - SA1 / SA2 and prevents this way the loss of centromeric cohesin. If SgoI gets phosphorylated centromeric cohesin becomes removed (Hauf et al., 2005; Yamagishi et al., 2008). Plk1 and not Aurora B seems to be required for dissociation of cohesion from

chromosome arms in early mitosis by triggering phosphorylation of the cohesin subunits Scc1 and SA2 (Hauf et al., 2005).

In my opinion the by Soung *et al.* observed recruitment of Plk1 by Odf2 / Cenexin is related to centriole disengagement and not to chromosomal segregation defects (Soung et al., 2006). Odf2 / Cenexin may connect both centrioles of the centrosome with its fiber forming capability and the recruitment of Plk1 is vital to phosphorylate Shugoshin (sSgoI) and allow the separation of centrioles (Tsang and Dynlacht, 2008; Wang et al., 2006; Wang et al., 2008). Corollary will be multiple centrosomes and multiple spindles, which possibly can be analyzed by microscopy.

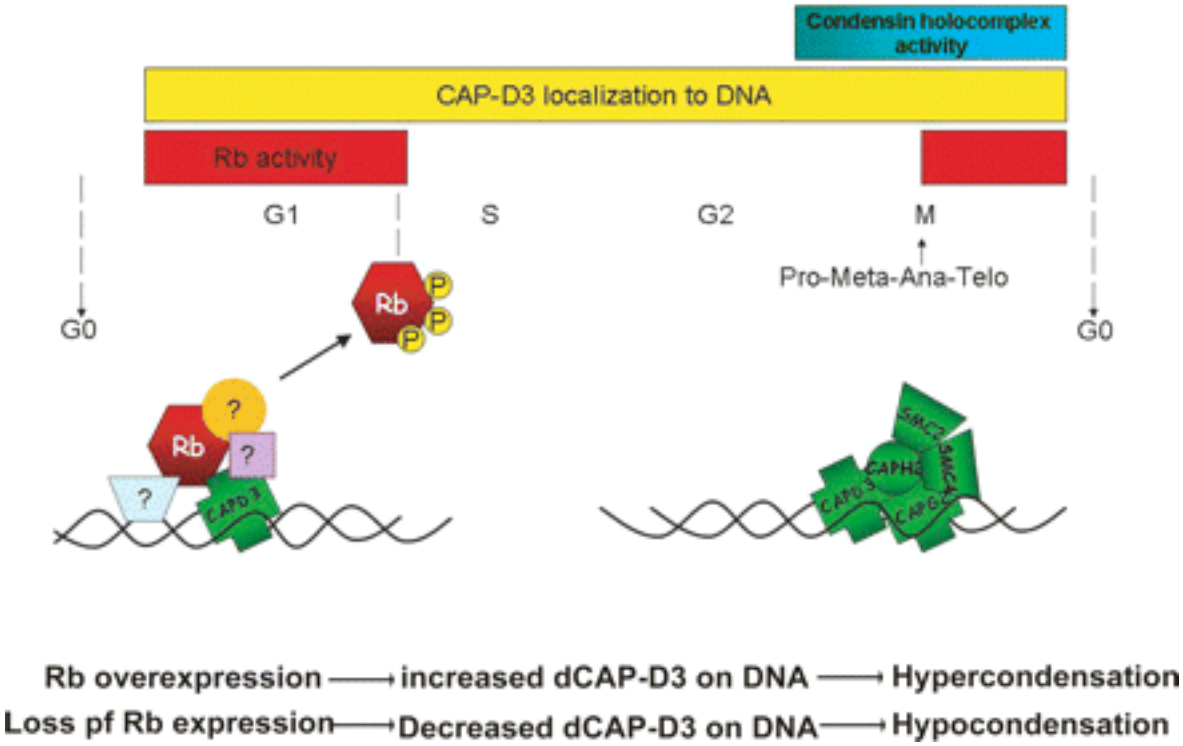

Figure 7 (Longworth et al., 2008): Members of the Rb family can interact with CAP-D3 and promoting its association to chromatin. Odf2 / Cenexin RNAi leads to a decrease of the Rb level and to a decrease of the condensing complex component CAP-D3 resulting in hypocondensation of chromosomes. Hypocondensation is caused by H3S10ph decrease as well.

Further work that elucidates the influence of Odf2 / Cenexin on the condensin complex will be necessary to support my theory that Odf2 / Cenexin is involved in chromosome condensation by influencing H3S10 phosphorylation. In summary my findings fit the working model established by Longworth *et al.* comprising the result of Rb level determined by western blot after Odf2 / Cenexin RNAi or overexpression. *Drosophila* RBF1 overexpression leads to an increased association dCAP-D3 with chromatin and results in hypercondensation of chromosomes whereas a loss of RBF1 expression causes hypocondensation. Odf2 / Cenexin RNAi leads to a decrease of cellular Rb levels. This in turn causes a decreased

association of CAP-D3 with chromatin and results in hypocondensation of chromosomes. The loss of the H3S10ph mark could trigger hypocondensation and therefore lead to inefficient resolution of chromatids, anaphase defects such as lagging chromosomes and chromosome bridges (Lam et al., 2006; Savvidou et al., 2005) and finally cause aneuploidy and cancer.

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6. Abbreviations

γTuRC	γ-tubulin ring complex
°C	degree centigrade
aa	amino acid
amp	ampère
APC	anaphase promoting complex
bp	basepaire
BSA	bovine serum albumine
cdk	cyclin – dependent kinase
cDNA	complementary DNA
DAPI	4´6-Diamino-2-phenylindoldihydrochlorid
Dm	*Drosophila melanogaster*
DMEM	Dulbecco´s Modified Eagles Medium
DNA	deoxyribonucleic acid
dNTPs	deoxynucleotides
DTT	Dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EGFP	enhanced green fluorescent protein
ELM	Eukaryotic Linear Motif Resource for Functional Sites in Proteins
et al.	et alteres; et alii
FACS	fluorescence activated cell sorting
FBS	fetal bovine serum
fig.	figure
g	gram
g	gravitation
G1	1^{st} gap phase
G2	2^{nd} gap phase
GSSG	oxidized glutathione
GSSH	reduced glutathione
GST	gluthadione-S-transferase
h	hour
H1	histone 1; linker histone
H2A	histone 2A
H2B	histone 2B

H3	histone 3
H3K9me2	dimethylation of lysine 9 at histone 3
H3K9me3	trimethylation of lysine 9 at histone 3
H3S10ph	phosphorylation of serin 10 at histone 3
H4	histone 4
HA	hemagglutinin
HP1	heterochromatin protein 1
kb	kilobase
kDa	kilodalton
LB-Medium	Laura-Bertani-Medium
LSM	laser scanning microscope
m	milli
M	molar
min	minutes
ml	milliliter
mm	milimeter
mM	millimolar
Mm	*Mus musculus*
MT	microtubuli
MTOC	microtubuli organizing centre
ng	nanogram
nm	nanometer
OD	optical density
Odf2	outer dense fiber protein 2
PBS	phosphate buffered saline
PCM	pericentriolar matrix
PCR	polymerase chain reaction
pH	potentium hydrogenii
Plk1	Polo - like kinase 1
PMSF	phenylmethanesulphonylfluoride
pp.	paginae
Rb	Retinoblastoma protein
Rn	*Rattus norwegicus*
RNA	ribonucleic acid
RNAi	RNA interference
rpm	rounds per minute
RT	room temperature

RT-PCR	reverse transcription polymerase chain reaction
S phase	synthesis phase
sec	seconds
Sgo1	Shugoshin 1
siRNA	small interfering RNA, short interfering RNA
smc	structural maintenance of chromosomes
tab.	table
U	Units
v/v	volume/volume
w/v	weight/volume
x	times
ZW10	Zeste White 10
Zwint-1	ZW10 interacting protein 1
µ	micro
µg	microgram
µM	micromolar

Amino acids and nucleic acids are shortened in according to the international one – letter - codes.

7. Appendix

A: Amino acid sequences alignment of Rb mutants used in this study

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 1                                                            60
  Rb  MPPKTPRKTA ATAAAAAAEP PAPPPPPPPE EDPEQDSGPE DLPLVRLEFE ETEEPDFTAL
Rb36  MPPKTPRKTA ATAAAAAAEP PAPPPPPPPE EDPEQDSGPE DLPLVRLEFE ETEEPDFTAL
 Rb9  MPPKTPRKTA ATAAAAAAEP PAPPPPPPPE EDPEQDSGPE DLPLVRLEFE ETEEPDFTAL
Rb63  MPPKTPRKTA ATAAAAAAEP PAPPPPPPPE EDPEQDSGPE DLPLVRLEFE ETEEPDFTAL

      61                                                    120
  Rb  CQKLKIPDHV RERAWLTWEK VSSVDGVLGG YIQKKKELWG ICIFIAAVDL DEMSFTFTEL
Rb36  CQKLKIPDHV RERAWLTWEK VSSVDGVLGG YIQKKKELWG ICIFIAAVDL DEMSFTFTEL
 Rb9  CQKLKIPDHV RERAWLTWEK VSSVDGVLGG YIQKKKELWG ICIFIAAVDL DEMSFTFTEL
Rb63  CQKLKIPDHV RERAWLTWEK VSSVDGVLGG YIQKKKELWG ICIFIAAVDL DEMSFTFTEL

      121                                                   180
  Rb  QKNIEISVHK FFNLLKEIDT STKVDNAMSR LLKKYDVLFA LFSKLERTCE LIYLTQPSSS
Rb36  QKNIEISVHK FSNLLKEIDT STKVDNAMSR LLKKYDVLFA LFSKLERTCE LIYLTQPSSS
 Rb9  QKNIEISVHK FFNLLKEIDT STKVDNAMSR LLKKYDVLFA LFSKLERTCE LIYLTQPSSS
Rb63  QKNIEISVHK FFNLLKEIDT STKVDNAMSR LLKKYDVLFA LFSKLERTCE LIYLTQPSSS

      181                                                   240
  Rb  ISTEINSALV LKVSWITFLL AKGEVLQMED DLVISFQLML CVLDYFIKLS PPMLLKEPYK
Rb36  ISTEINSALV LKVSWITFLL AKGEVLQMED DLVISFQLML CVLDYFIKLS PPMLLKEPYK
 Rb9  ISTEINSALV LKVSWITFLL AKGEVLQMED DLVISFQLML CVLDYFIKLS PPMLLKEPYK
Rb63  ISTEINSALV LKVSWITFLL AKGEVLQMED DLVISFQLML CVLDYFIKLS PPMLLKEPYK

      241                                                   300
  Rb  TAVIPINGSP RTPRRGQNRS ARIAKQLEND TRIIEVLCKE HECNIDEVKN VYFKNFIPFM
Rb36  TAVIPINGSP RTPRRGQNRS ARIAKQLEND TRIIEVLCKE HECNIDEVKN VYFKNFIPFM
 Rb9  TAVIPINGSP RTPRRGQNRS ARIAKQLEND TRIIEVLCKE HECNIDEVKN VYFKNFIPFM
Rb63  TAVIPINGSP RTPRRGQNRS ARIAKQLEND TRIIEVLCKE HECNIDEVKN VYFKNFIPFM

      301                                                   360
  Rb  NSLGLVTSNG LPEVENLSKR YEEIYLKNKD LDARLFLDHD KTLQTDSIDS FETQRTPRKS
Rb36  NSLGLVTSNG LPEVENLSKR YEEIYLKNKD LDARLFLDHD KTLQTDSIDS FETQRTPRKS
 Rb9  NSLGLVTSNG LPEVENLSKR YEEIYLKNKD LDARLFLDHD KTLQTDSIDS FETQRTPRKS
Rb63  NSLGLVTSNG LPEVENLSKR YEEIYLKNKD LDARLFLDHD KTLQTDSIDS FETQRTPRKS

      361                                                   420
  Rb  NLDEEVNVIP PHTPVRTVMN TIQQLMMILN SASDQPSENL ISYFNNCTVN PKESILKRVK
Rb36  NLDEEVNVIP PHTPVRTVMN TIQQLMMILN SASDQPSENL ISYFNNCTVN PKESILKRVK
 Rb9  NLDEEVNVIP PHTPVRTVMN TIQQLMMILN SASDQPSENL ISYFNNCTVN PKESILKRVK
Rb63  NLDEEVNVIP PHTPVRTVMN TIQQLMMILN SASDQPSENL ISYFNNCTVN PKESILKRVK

      421                                                   480
  Rb  DIGYIFKEKF AKAVGQGCVE IGSQRYKLGV RLYYRVMESM LKSEEERLSI QNFSKLLNDN
Rb36  DIGYIFKEKF AKAVGQGCVE IGSQRYKLGV RLYYRVMESM LKSEEERLSI QNFSKLLNDN
 Rb9  DIGYIFKEKF AKAVGQGCVE IGSQRYKLGV RLYYRVMESM LKSEEERLSI QNFSKLLNDN
Rb63  DIGYIFKEKF AKAVGQGCVE IGSQRYKLGV RLYYRVMESM LKAAEERLSI QNFSKLLNDN

      481                                                   540
  Rb  IFHMSLLACA LEVVMATYSR STSQNLDSGT DLSFPWILNV LNLKAFDFYK VIESFIKAEG
Rb36  IFHMSLLACA LEVVMATYSR STSQNLDSGT DLSFPWILNV LNLKAFDFYK VIESFIKAEG
 Rb9  IFHMSLLACA LEVVMATYSR STSQNLDSGT DLSFPWILNV LNLKAFDFYK VIESFIKAEG
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Rb63  IFHMSLLACA LEVVMATYSR STSQNLDSGT DLSFPWILNV LNLKAFDFYK VIESFIKAEG

      541                                                        600
  Rb  NLTREMIKHL ERCEHRIMES LAWLSDSPLF DLIKQSKDRE GPTDHLESAC PLNLPLQNNH
Rb36  NLTREMIKHL ERCEHRIMES LAWLSDSPLF DLIKQSKDRE GPTDHLESAC PLNLPLQNNH
 Rb9  NLTREMIKHL ERCEHRIMES LAWLSDSPLF DLIKQSKDRE GPTDHLESAC PLNLPLQNNH
Rb63  NLTAEMIAHL ERCEHRIMES LAWLSDSPLF DLIKQSKDRE GPTDHLESAC PLNLPLQNNH

      601                                                        660
  Rb  TAADMYLSPV RSPKKKGSTT RVNSTANAET QATSAFQTQK PLKSTSLSLF YKKVYRLAYL
Rb36  TAADMYLSPV RSPKKKGSTT RVNSTANAET QATSAFQTQK PLKSTSLSLF YKKVYRLAYL
 Rb9  TAADMYLSPV RSPKKKGSTT RVNSTANAET QATSAFQTQK PLKSTSLSLF YKKVYRLAYL
Rb63  TAADMYLSPV RSPKKKGSTT RVNSTANAET QATSAFQTQK PLKSTSLSLF YAKVYALAYA

      661                                                        720
  Rb  RLNTLCERLL SEHPELEHII WTLFQHTLQN EYELMRDRHL DQIMMCSMYG ICKVKNIDLK
Rb36  RLNTLCERLL SEHPELEHII WTLFQHTLQN EYELMRDRHL DQIMMCSMYG ICKVKNIDLK
 Rb9  RLNTLCERLL SEHPELEHII WTLFQHTLQN EYELMRDRHL DQIMMCSMYG ICKVKNIDLK
Rb63  RLNALCEALL SEHPELEHII WTLFQHTLQN EYELMRDRHL DQIMMCSMYG ICKVKNIDLK

      721                                                        780
  Rb  FKIIVTAYKD LPHAVQETFK RVLIKEEEYD SIIVFYNSVF MQRLKTNILQ YASTRPPTLS
Rb36  FKIIVTAYKD LPHAVQETFK RVLIKEEEYD SIIVFYNSVF MQRLKTNILQ YASTRPPTLS
 Rb9  FKIIVTAYKD LPHAVQETFK RVLIKEEEYD SIAVFYASVF AQRLKTNILQ YASTRPPTLS
Rb63  FKIIVTAYKD LPHAVQETFK RVLIKEEEYD SIIVFYNSVF MQRLKTNILQ YASTRPPTLS

      781                                                        840
  Rb  PIPHIPRSPY KFPSSPLRIP GGNIYISPLK SPYKISEGLP TPTKMTPRSR ILVSIGESFG
Rb36  PIPHIPRSPY KFPSSPLRIP GGNIYISPLK SPYKISEGLP TPTKMTPRSR ILVSIGESFG
 Rb9  PIPHIPRSPY KFPSSPLRIP GGNIYISPLK SPYKISEGLP TPTKMTPRSR ILVSIGESFG
Rb63  PIPHIPRSPY KFPSSPLRIP GGNIYISPLK SPYKISEGLP TPTKMTPRSR ILVSIGESFG

      841                                                        900
  Rb  TSEKFQKINQ MVCNSDRVLK RSAEGSNPPK PLKKLRFDIE GSDEADGSKH LPGESKFQQK
Rb36  TSEKFQKINQ MVCNSDRVLK RSAEGSNPPK PLKKLRFDIE GSDEADGSKH LPGESKFQQK
 Rb9  TSEKFQKINQ MVCNSDRVLK RSAEGSNPPK PLKKLRFDIE GSDEADGSKH LPGESKFQQK
Rb63  TSEKFQKINQ MVCNSDRVLK RSAEGSNPPK PLAALRFDIE GSDEADGSKH LPGESKFQQK

      901                       928
  Rb  LAEMTSTRTR MQKQKMNDSM DTSNKEEK
Rb36  LAEMTSTRTR MQKQKMNDSM DTSNKEEK
 Rb9  LAEMTSTRTR MQKQKMNDSM DTSNKEEK
Rb63  LAEMTSTRTR MQKQKMNDSM DTSNKEEK
```

B: Raw data reportergeneassay

1st

1	2	3	average	%	
296,9	211,8	254,9	255	100%	endogenous
227,4	141,9	235,5	202	79%	overexpression of Odf2 /Cenexin
277,5	396,2	254,8	310	122%	Odf2 / Cenexin RNAi
221,6	264	182,8	223	87%	overexpression of retinoblastoma protein
166,5	219,1	189,9	192	75%	overexpression of retinoblastoma protein and Odf2 / cenexin
403	287	263,2	318	125%	overexpression of retinoblastoma protein and RNAi

2nd

1	2	3	average	%	
156	154		155	100%	endogenous
127	146	137	137	88%	overexpression of Odf2 /Cenexin
67	62	57	62	40%	Odf2 / Cenexin RNAi
119	110	118	116	75%	overexpression of retinoblastoma protein
135	135	128	133	86%	overexpression of retinoblastoma protein and Odf2 / cenexin
64	61	67	64	41%	overexpression of retinoblastoma protein and RNAi

1st	2nd		average	deviation
100%	100%	endogenous	100%	0
79%	88%	overexpression of Odf2 /Cenexin	84%	0,045
122%	40%	Odf2 / Cenexin RNAi	81%	0,41
87%	75%	overexpression of retinoblastoma protein	81%	0,06
75%	86%	overexpression of retinoblastoma protein and Odf2 / cenexin	81%	0,055
125%	41%	overexpression of retinoblastoma protein and RNAi	83%	0,42

C: Alamar Blue data

	siRNA	p13.8NCgfp	pEGFP-N1	control	p13.8NCEgfp	pRB-HA	pRB-HA + siRNA	pRB-HA + p13.8NCEgfp	ODF2N2C	ODF2N2C + pRB-HA	siLentFect	Transfectin
24h	0,047	-0,013	0,036	0,054	0,031	0,036	0,04	0,035	0,036	0,032	0,042	0,04
	0,049	0,034	0,051	0,058	0,035	0,037	0,017	0,037	0,035	0,041	0,042	0,134
	4,00E-03	0,053	0,045	0,074	0,033	0,034	0,039	0,034	0,035	0,088	0,042	0,04
	0,051	0,04	0,047	0,059	0,039	0,033	0,04	0,037	0,037	0,037	0,049	-0,023
	0,055	0,038	0,035	0,076	0,034	0,034	0,037	-0,023	0,038	0,043	0,043	0,04
	0,051	0,039	0,042	0,054	0,036	0,035	0,037	0,036	0,036	0,04	0,052	0,043
average	0,042833333	0,031833333	0,042666667	0,0625	0,034666667	0,034833333	0,035	0,026	0,217	0,046833333	0,045	0,045666667
normalized	0,685333333	0,509333333	0,682666667	1	0,99047619	0,55728	0,56	0,416	3,472	0,749333333	0,72	0,730666667
stdev	0,019208505	0,022885949	0,00628225	0,009914636	0,00273252	0,00147196	0,008921883	0,02403331	0,001169045	0,020527218	0,00438178	0,050242081
48h	0,071	0,032	0,034	0,029	0,029	0,03	0,04	0,031	0,036	0,03	0,036	0,047
	0,083	0,03	0,033	0,099	0,032	0,03	0,036	0,035	0,037	0,035	0,038	0,036
	0,069	0,034	0,034	0,105	0,032	0,029	0,037	0,031	0,034	0,033	0,031	0,052
	0,066	0,036	0,036	0,108	0,033	0,032	0,037	0,034	0,035	0,034	0,042	0,03
	0,172	0,036	0,033	0,101	0,036	0,031	0,035	0,039	0,038	0,037	0,039	0,044
	0,06	0,038	0,035	0,11	0,036	0,033	0,038	0,032	0,035	0,039	0,038	0,047
	0,054	0,032	0,034	0,101	0,03	0,031	0,039	0,023	-0,035	0,028	0,062	0,048
	0,049	0,032	0,032	0,108	0,035	0,028	0,038	0,039	0,037	0,035	0,046	0,045
	0,06	0,038	0,038	0,164	0,033	0,027	0,038	0,034	0,045	0,037	0,056	0,047
	0,068	0,036	0,064	0,085	0,053		0,037	0,052	0,036	0,034	0,057	0,041
	0,063	0,034	0,032	0,091	0,036	0,031	0,041	0,032	0,035	0,034	0,061	0,041
	0,058	0,041	0,038	0,107	0,036	0,032	0,041	0,036	0,037	0,036	0,056	0,051
average	0,07275	0,034916667	0,036916667	0,100666667	0,035083333	0,030363636	0,038083333	0,034833333	0,030833333	0,034333333	0,046833333	0,044083333
normalized	1,164	0,558666667	0,590666667	1,610666667	1,002380952	0,485818182	0,609333333	0,557333333	0,493333333	0,549333333	0,749333333	0,705333333
stdev	0,032465716	0,003175426	0,008764166	0,029760661	0,006141636	0,001804036	0,001880925	0,006860073	0,020923381	0,003025147	0,010919818	0,006287915
72h	0,984	0,968	0,939	1,194	0,951	0,949	0,98	0,939	0,974	0,954	0,977	0,979
	0,987	0,952	0,952	1,213	0,968	0,946	0,963	0,957	0,961	0,965	0,984	0,963
	1,165	0,961	0,966	1,199	0,965	0,951	0,965	1,379	0,966	0,965	0,976	0,984
	1,001	0,981	0,982	1,214	0,962	0,955	0,967	0,966	0,96	0,962	0,994	0,969
	1,121	0,975	0,942	1,174	0,967	0,953	0,958	0,953	0,975	0,985	0,993	0,977
	0,975	0,978	0,962	1,189	0,961	0,962	0,959	0,952	0,96	0,962	0,981	1,004
	1,036	0,967	0,955	1,149	0,951	0,959	0,968	0,954	0,964	0,961	1,089	0,984
	1,06	0,954	0,958	1,14	0,966	0,943	0,957	0,956	0,961	0,962	1,069	0,965
	1,035	0,965	0,962	1,172	0,958	0,953	0,966	0,937	0,892	0,809	0,976	1,01
	1,022	0,962	0,981	1,179	0,959	0,958	0,963	0,967	0,964	0,963	1,049	1,068
	1,049	0,953	0,955	1,191	0,958	0,954	0,957	0,944	0,981	0,972	1,077	0,995
	1,02	0,974	0,963	1,197	0,961	0,964	0,965	0,957	0,955	0,956	1,069	1,021
average	1,037916667	0,965833333	0,95975	1,18425	0,960583333	0,953916667	0,964	0,988416667	0,959416667	0,951333333	1,0195	0,99325
normalized	16,60666667	15,45333333	15,356	18,948	27,4452381	15,26266667	15,424	15,81466667	15,35066667	15,22133333	16,312	15,892
stdev	0,056464725	0,009861157	0,01300437	0,022839858	0,00561586	0,006229816	0,006381792	0,123349067	0,022516492	0,045519892	0,046312182	0,029689683

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11/2006 – 01/2009	Dr. rer. nat. (PhD) at the Göttingen Centre for Molecular Biosciences (GZMB), Georg – August – University Göttingen (Germany), supervised by Prof. Dr. Sigrid Hoyer – Fender. Grade: magna cum laude Dissertation topic: “Cellular and Biochemical Analysis of an Outer Dense Fiber Protein 2 (Odf2) Variant and the Endogenous Odf2 / Cenexin in Functional Approaches”.
04/2006- 10/2006	Internship at the German Cancer Research Centre (DKFZ), Heidelberg (Germany). Junior Research Group Molecular Biology of Centrosomes and Cilia.

2005 Diploma in Biology at the University of Göttingen (Germany). (Grade: gut (B)) *Major exam subjects:* developmental biology, immunobiology, microbiology.

Diploma thesis:
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Hüber, D., and S. Hoyer-Fender. 2007. Alternative splicing of exon 3b gives rise to ODF2 and Cenexin. Cytogenet Genome Res. 119:68-73.

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