DOC1R: a MAP kinase substrate that control microtubule organization of metaphase II mouse oocytes

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Summary

For the success of fertilization, spindles of vertebrate oocytes must remain stable and correctly organized during the arrest in metaphase II of meiosis. Using a two-hybrid screen with MAPK as a bait, we have recently identified MISS (MAPK interacting and spindle stabilizing) which controls mouse oocyte metaphase II spindle stability. Using the same screen, we identify another MAPK partner, DOC1R (Deleted in oral cancer one related), a murine homologue of a potential human tumor suppressor gene. We characterize DOC1R during mouse oocyte meiosis resumption. DOC1R is regulated by phosphorylation during meiotic maturation by MPF (M-phase promoting factor) and by the MOS/…/MAPK pathway. DOC1R and a DOC1R-GFP fusion localize to microtubules during meiotic maturation. Consistent with this microtubular localization, we show, by antisense and double-stranded RNA injection, that depletion of DOC1R induces microtubule defects in metaphase II oocytes. These defects are rescued by overexpressing a Xenopus DOC1R, showing that they are specific to DOC1R. Thus, the discovery of DOC1R, a substrate of MAPK that regulates microtubule organization of metaphase II mouse oocytes, reinforces the importance of this pathway in the control of spindle stability during the metaphase II arrest.

Key words: DOC1R, MAPK, Mouse meiotic maturation, Microtubules

Introduction

After ovulation, mouse oocytes arrest for several hours in metaphase II (MII) of the second meiotic division with a stable spindle. Typically, mouse meiotic spindles are barrel shaped and are devoid of astral microtubules. These meiotic spindles organize from MTOCs (microtubule organizing centers) that lack centrioles. During the arrest in metaphase II, chromosomes remain aligned on the metaphase plate, waiting for fertilization to trigger chromosome segregation. Not much is known about the precise mechanisms that allow the second meiotic spindle to remain correctly organized during the so-called CSF (cytostatic factor) (Masui and Markert, 1971) arrest in metaphase II of vertebrate oocytes. This CSF arrest is mediated by the MOS/…/MAPK (mitogen activated protein kinase) pathway and has been mainly studied on the aspect of MPF (M-phase promoting factor) (Masui and Markert, 1971) stabilization. Indeed, during the metaphase II arrest, MPF (a complex between CDC2 and cyclin B) levels remain high, owing to the CSF activity generated by the MOS/…/MAPK pathway (Colledge et al., 1994; Dupre et al., 2002; Haccard et al., 1993; Hashimoto et al., 1994; Nebreda and Hunt, 1993; Posada et al., 1993; Verlhac et al., 1996). In *Xenopus* oocytes, MAPK phosphorylates p90rsk which in turns phosphorylates Bub1, which would inhibit the APC/C (anaphase promoting complex/cyclosome) to target cyclin B for degradation therefore maintaining a high MPF activity (Gross et al., 1999; Gross et al., 2000; Tunquist et al., 2002). However, the MOS/.../MAPK pathway also controls microtubule organization, as mouse *Mos*$^{-/-}$ oocytes are not arrested in metaphase II, instead they activate spontaneously and remain arrested in a third metaphase with monopolar spindles (Verlhac et al., 1996). Furthermore, using a two-hybrid screen with MAPK as a bait on a mouse oocyte cDNA library, we have recently identified MISS (MAPK interacting and spindle stabilizing) a substrate of the MOS/…/MAPK pathway which mediates metaphase II spindle stability during the CSF arrest (Lefebvre et al., 2002). For the success of fertilization and future embryo development, it is therefore a key issue to understand at a cellular level how the MOS/…/MAPK pathway maintains a spindle correctly organized in metaphase II.

We describe the isolation of another MAPK partner using the same two-hybrid screen: DOC1R (D19ERTD144E – Mouse Genome Informatics), a murine homologue of a potential human tumor suppressor gene DOC1R (deleted in oral cancer one related) (Zhang et al., 1999). Until now, little was known about this protein that has been found on the basis of its homology with human DOC1, a human tumor suppressor gene (Todd et al., 1995). DOC1R is related to a coiled-coil region of a kinesin (KIF14) of unknown function. Like MISS, DOC1R is rich in proline residues in its N terminus. DOC1R has a perfect consensus site for MAPK phosphorylation, a potential CDK2 binding site and a potential cyclin/CDK binding site (Zhang et al., 1999). We show here that DOC1R

has been conserved from *Xenopus laevis* to human, which suggests that it performs important functions in vertebrate species. In mouse oocytes, DOC1R is present at all stages of meiotic maturation and is regulated by multiple phosphorylations. Both cyclin B/CDC2 and MAPK are able to phosphorylate DOC1R in vitro, and the MOS/…/MAPK pathway phosphorylates DOC1R in vivo. This protein is localized in dots on microtubules especially on metaphase I and II spindles. Consistently, a DOC1R-GFP fusion localizes to the metaphase II spindle. The depletion of DOC1R by microinjection of antisense (asRNA) or double-stranded (dsRNA) RNA directed against its endogenous mRNA has a strong effect on the metaphase II spindle morphology. Injected oocytes harbor spindles with astral microtubules, as well as numerous asters in the cytoplasm, suggesting that DOC1R regulates microtubule organization during the CSF arrest of metaphase II oocytes. We show that this phenotype is specific to DOC1R as it can be rescued by the overexpression of the *Xenopus* protein. Thus, we have discovered a new class of proline-rich proteins, MISS and DOC1R, substrates of MAPK that regulate microtubule organization during the CSF arrest of mouse oocytes.

Materials and methods

Collection and culture of mouse oocytes

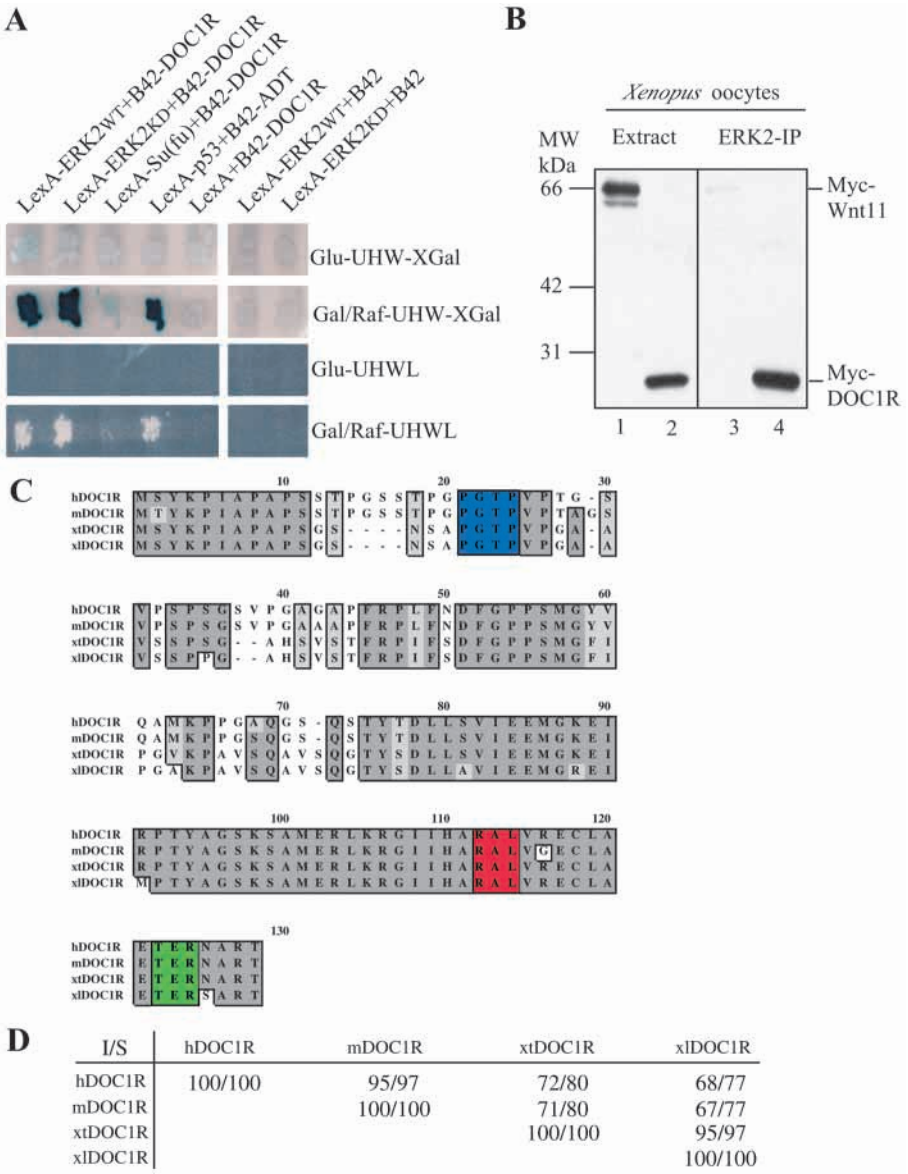
Immature oocytes arrested in prophase I of meiosis were obtained by removing ovaries from 11-week-old OF1 and *Mos*$^{-/-}$ female mice. Oocytes were removed and cultured as previously described (Verlhac et al., 1996). Nocodazole was diluted in the culture medium at 10 μM.

Two-hybrid screen

The two-hybrid screen was performed as previously described (Lefebvre et al., 2002).

RT-PCR assay

For preparing RNA from mouse ovaries, total RNA were extracted using the Rneasy mini Kit (Qiagen). Immature mouse oocytes were lysed in PBS without total RNA extraction. Then 500 ng of RNA from ovaries, or 30 immature oocytes in sterile PBS were treated with 2 U of RQ1 DNAse (Promega) for 20 minutes at 37°C and heated for 5 minutes at 85°C. The first strand cDNA synthesis was performed with 50 U of MmuLV Superscript (Life Technologies) using 2.5 μM random hexamer (pdN6, Pharmacia), 1 mM dNTPs



I/S	hDOC1R	mDOC1R	xtDOC1R	xlDOC1R
hDOC1R	100/100	95/97	72/80	68/77
mDOC1R		100/100	71/80	67/77
xtDOC1R			100/100	95/97
xlDOC1R				100/100

Fig. 1. Interaction between DOC1R and MAPK; alignments of DOC1R sequences. (A) Two-hybrid interaction between DOC1R and MAPK. DOC1R interacts with ERK2WT and ERK2KD but not with the negative control Su(Fu). Yeast strains transformed with LexA-ERK2WT, LexA-ERK2KD (Waskiewicz et al., 1997), LexA-53 (positive control), LexA-Su(Fu) or LexA alone were mated with yeast strains transformed respectively with the B42-DOC1R, B42-AD-T (positive control), or empty B42. The diploids obtained were tested for transactivation of both the β-galactosidase and the LEU2 reporter genes on glucose (Glu)- or galactose (Gal/Raf)-containing mediums. The B42 constructs are under the control of the galactose promoter. The B42-DOC1R fusion protein clearly interacts both with LexA fusions of ERK2WT and ERK2KD as strongly as the positive control, whereas it does not interact with the negative control Su(Fu). (B) DOC1R co-immunoprecipitates with endogenous p42^{mapk} (ERK2) from immature *Xenopus* oocyte extracts. Lanes 1 and 2: total immature *Xenopus* oocyte extracts expressing either MYC-WNT11 (*Xenopus* WNT11, a negative control, lane 1) or MYC-DOC1R mRNA (lane 2). Lanes 3 and 4: anti-p42^{mapk} (ERK2) immunoprecipitates prepared from the MYC-WNT11 (lane 3) and MYC-DOC1R (lane 4) expressing oocyte lysates. All samples were analysed by immunoblotting using the anti-MYC antibody. This experiment was repeated twice. (C) Amino acid sequence alignments of the DOC1R protein from different vertebrate species. DOC1R is rich in proline in its N-terminal end and contains one potential MAPK phosphorylation site (blue), one CDK2 binding site (red) and one cyclin/CDK-binding site (green). (D) Percentage of identities (I) and similarities (S) between the amino acid sequences of DOC1R from different vertebrate species (h, human; m, mouse; xt, *Xenopus tropicalis*; xl, *Xenopus laevis*).

(Promega), in 10 mM Tris-HCl pH 8.3, 5 mM $MgCl_2$, 50 mM KCl for 1 hour at 37°C, followed by 5 minutes at 95°C. The PCR amplification was performed using 5′-ATGCCTCGAGATGACG-TACAAGCCAATCGC and 5′-ATGCGAATTCCCGTGCGGGCA-TTGCGTTCT primers, at 55°C for 30 cycles.

Plasmid construction and in vitro synthesis of capped RNA, asRNA and dsRNA

The pRN3MYC2-DOC1R was constructed by RT-PCR amplification of the DOC1R open reading frame from RNA isolated from mouse ovaries. Total RNA were extracted using the Rneasy Mini Kit (Qiagen). The reverse transcription was performed on 500 ng of RNA. The PCR amplification was done on 50 ng of RNA/DNA using 5′-GATCGAATTCATGWSNTAYAARCCNATHGCN and 5′-GATC-GCGGCCGCTTACGTGCGGGCATTGCGTTC primers. The PCR product was then cloned into the pRN3MYC2 vector. The pRN3DOC1R-GFP was obtained by PCR subcloning at *Xho*I/*Eco*RI sites using 5′-ATGCCTCGAGATGACGTACAAGCCAATCGC and 5′-ATGCGAATTCCCGTGCGGGCATTGCGTTCT primers. The pET-DOC1R plasmid was constructed by subcloning at *Eco*RI/*Not*1 sites into the pET30a vector (Novagen). The DOC1R protein expressed from the pET30a vector contains a 6His repeat inside 51 additional amino acids, which makes it bigger of about 5 kDa.

The *Xenopus* cDNA was isolated from the *Xenopus* oocyte cDNA library (Iouzalem et al., 1998) by PCR using the 5′-ATGCGA-ATTCATGTCGTATAAACCAT and 3′-ATGCGCGGCCGCTCATG-TGCGGGCACTGCGTTCTGT primers on 200 ng of library. The PCR product was further cloned into pRN3 at the *Eco*RI/*Not*I sites.

The in vitro synthesis of capped RNA, asRNA and dsRNA was performed using linearized plasmids with the mMessage mMachine kit for capped RNAs (Ambion) or with the Megascript Kit for as and dsRNA (Ambion). The capped RNAs and asRNA were then purified on RNeasy columns (Qiagen) and eluted in the injection buffer (10 mM Tris, 0.1 mM EDTA, pH 7.4) at a final concentration of 0.5 μg/μl. Aliquots were then stored at –80°C. For the production of dsRNA, each strand of complementary RNA was first precipitated with ethanol then washed in phenol/chloroform and further incubated at 85°C for 5 minutes. After annealing for 3 hours at 37°C, 4 μl aliquots of dsRNA were stored at – 80°C.

Microinjection of synthetic RNA

Microinjection into mouse oocytes was performed as described (Verlhac et al., 2000).

Co-immunoprecipitation in Xenopus oocyte extracts

Xenopus oocyte microinjection as well as oocyte extraction was performed as previously described (Gavin et al., 1999). Samples of 30 oocytes were extracted in 300 μl of lysis buffer (80 mM β-glycerophosphate, pH 7.4, 20 mM EGTA, 15 mM $MgCl_2$, 100 μg/ml leupeptin, 100 μg/ml aprotinin, 1 mM sodium orthovanadate, 2 mM PMSF). The oocyte extract was then clarified by centrifugation at 13,000 rpm at 4°C for 20 minutes. The supernatant was removed from the overlying lipid layer and the yolk protein and was further clarified by a second centrifugation at 13,000 rpm at 4°C for 10 minutes. The oocyte extracts were stored at –70°C. For the immunoprecipitation of endogenous $xp42^{mapk}$, we used an anti-ERK2 antibody conjugated to agarose (Santa Cruz Biotechnology). *Xenopus* oocyte extracts (300 μl) were pre-cleared with 20 μl of protein A coupled to agarose beads for 30 minutes at 4°C. The cleared extracts were then incubated for 2 hours at 4°C with the antibody coupled to 20 μl of agarose beads. The beads were washed four times in 1 ml lysis buffer supplemented with 150 mM NaCl and then processed for immunoblotting.

Dephosphorylation assay

Just after collection and lysis, oocytes were incubated with or without 400 U of λ-phosphatase (New England Biolabs) in λ-phosphatase buffer at 37°C for 1 hour and then processed for immunoblotting.

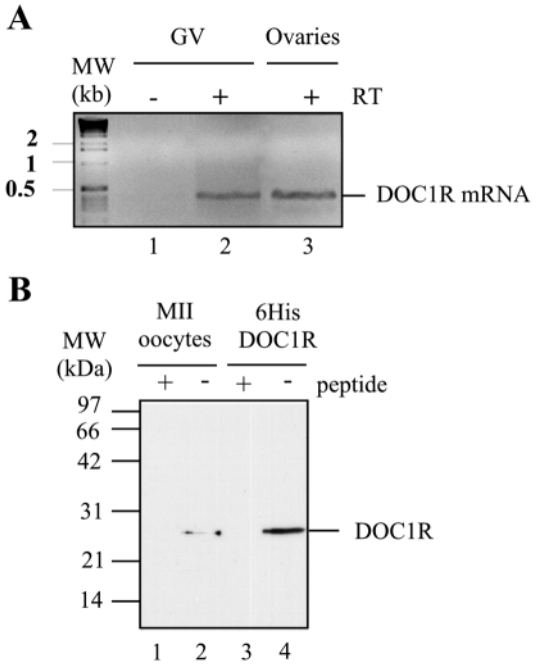


Fig. 2. DOC1R mRNA and protein are present in mouse oocyte. (A) Expression of DOC1R mRNA in immature oocytes (lanes 1 and 2) and ovaries (lane 3), treated or not with reverse transcriptase (RT, + or –). (B) The anti-DOC1R antibody recognizes a band at 28 kDa in 200 oocytes arrested in metaphase II (lane 2) and recognizes 1 μg of purified DOC1R protein (lane 4). The antibody pre-incubated with the DOC1R peptide (peptide, + or –) does not recognize either endogenous DOC1R (lane 1) or purified DOC1R (lane 3). These experiments were repeated twice.

Preparation of recombinant DOC1R protein

The DOC1R recombinant protein was prepared from the HMS174 *E. coli* transformed with the pET-DOC1R plasmid. The protein was purified from bacteria under native conditions as described in the QIAexpressionist handbook (Qiagen).

Kinase assays

The in vitro kinase assays were performed on 0.1 μg of purified DOC1R or 2.5 μg of Histone H1 in 10 μl of Histone H1 kinase buffer [80 mM β-glycerophosphate, 20 mM EGTA, 15 mM $MgCl_2$, 2 μg/ml leupeptin, 2 μg/ml aprotinin, 2.5 mM benzamidine, 1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride and 1 mM DTT, pH 7.4] with 0.025 μg of commercial recombinant active rat ERK2 (BIOMOL, Ref SE-137) or 0.025 μg of cyclin B/CDC2 (Biolabs) supplemented with 6.25 μCi of [γ-^{32}P]-ATP (3000 Ci/mmole) and 50 μM cold ATP, for 30 minutes at 37°C. Reactions were stopped by the addition of sample buffer (Laemmli, 1970) and were analyzed by SDS-PAGE followed by autoradiography.

2D gel electrophoresis

Oocytes microinjected with MYC-DOC1R encoding RNA were collected 14 hours after GVBD and stored at –80°C in water containing 1 mM PMSF and 10 μg/ml leupeptin/pepstatin/aprotinin. They were then processed as previously described (Louvet-Vallee et al., 2001).

Immunocytochemistry

Immunocytochemistry was performed as previously described (Brunet et al., 1999). To visualize endogenous DOC1R, oocytes were first fixed for 30 minutes in 3.7% formaldehyde at 30°C then treated

for 10 minutes in 0.25% TritonX-100 in PBS, washed in 0.1% Tween 20/PBS, incubated for 1 hour with the primary affinity-purified anti-DOC1R antibody (at 1:50) in 3% BSA/0.1% Tween 20/PBS. They were then washed in 0.1% Tween 20/PBS and further incubated for 1 hour with the secondary anti-rabbit FITC (1:80) in 3% BSA/0.1% Tween 20/PBS. After incubation with the secondary antibody, all samples were washed in 0.1% Tween 20/PBS, incubated for 5 minutes in Propidium Iodide (5 μg/ml in 0.1%Tween20/PBS), then washed three times in PBS before mounting in Citifluor (Chem. Lab., UCK).

Immunoblotting

Oocytes at the appropriate stage of maturation were collected in sample buffer (Laemmli, 1970) and heated for 3 minutes at 100°C. We used the following antibodies: an affinity-purified anti-DOC1R antibody directed against the LVRECLAETERNART peptide (Sigma Immunochemicals), the anti-MYC 9E10 monoclonal antibody (sc-40; Santa Cruz Biotechnology), an anti-Ezrin antibody (Louvet-Vallee et al., 2001) and the anti-Erk antibody (sc-94; Santa Cruz Biotechnology).

Results

Isolation of DOC1R, a MAPK-interacting protein

MISS was isolated from a two-hybrid screen using rat ERK2 as a bait on a mouse oocyte cDNA library that we had prepared in the laboratory (Lefebvre et al., 2002). During the same screen, we had identified another partial cDNA of about 500 bp encoding a protein domain that interacts with rat ERK2. The homology of this partial cDNA with the human DOC1R (Zhang et al., 1999) coding sequence allowed us to isolate the full-length ORF of the murine DOC1R. The full-length DOC1R protein specifically interacts with ERK2 in a two-hybrid assay, as yeast strains co-transformed with plasmids expressing LexA-ERK2 and B42-DOC1R can induce expression of the two reporter genes (Leu2 and β-galactosidase; Fig. 1A). A MYC-tagged DOC1R protein also co-immunoprecipitates with endogenous MAPK from *Xenopus* oocyte extracts, whereas a negative control encoding for MYC-WNT11 does not (Fig. 1B compare lanes 3 and 4). All these data show that DOC1R is a MAPK partner. DOC1R, together with MISS (Lefebvre et al., 2002) and $p90^{rsk}$ (Kalab et al., 1996) are the only known MAPK substrates in mouse oocytes.

DOC1R potentially contains one MAPK phosphorylation site (Fig. 1C, blue), one CDK2 binding site (red) and a cyclin/CDK binding site (green) (Shintani et al., 2000). Like MISS, this protein is very rich in proline residues in its N-terminal end (Fig. 1C) (Lefebvre et al., 2002).

By homology searches in databases, we also isolated ESTs encoding homologues of the DOC1R protein (Fig. 1C). The DOC1R and human DOC1R protein are almost identical (they present 95% of identities, Fig. 1D). Interestingly, the protein is highly conserved from *Xenopus laevis* to human (about 70% of identities, Fig. 1D). This conservation suggests that DOC1R mediates important function(s) in vertebrate species.

DOC1R mRNA and protein are present in mouse oocytes

To investigate if the DOC1R mRNA was expressed in mouse ovaries and oocytes, we performed RT-PCR using specific primers on total RNA from ovaries and immature oocytes. The presence of the DOC1R mRNA in ovaries and oocytes is shown in Fig. 2A (lanes 2 and 3).

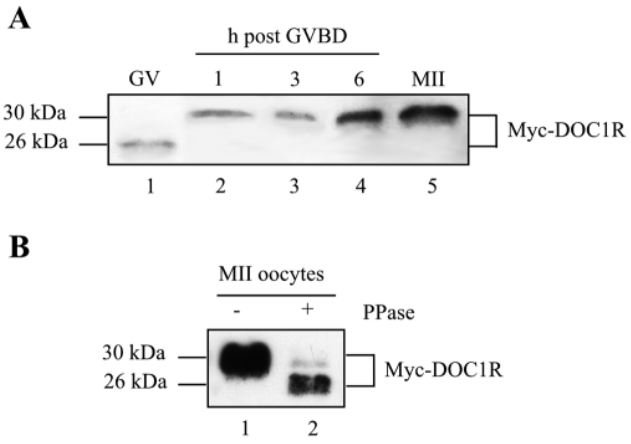


Fig. 3. DOC1R is regulated by phosphorylation. For A,B, immature oocytes were injected with RNA encoding the MYC-DOC1R protein and further cultured for different length of time. Each sample corresponds to a pool of 25 oocytes from the same injection and to oocytes that expressed the mRNA for at least 6 hours. (A) MYC-DOC1R regulation during meiotic maturation. MYC-DOC1R mRNA was injected into wild-type oocytes that were collected at different stages of meiotic maturation: immature (lane 1, GV), at 1 hour (lane 2), 3 hours (lane 3), 6 hours (lane 4) and 14 hours (lane 5, Metaphase II, MII) after GVBD. (B) In vitro dephosphorylation of MYC-DOC1R from mouse oocyte extracts. Thirty oocytes were injected with MYC-DOC1R mRNA, cultured 14 hours after GVBD, collected and incubated without (lane 1) or with (lane 2) 400 U of λ-phosphatase (PPase + or –). All samples were analyzed by immunoblotting with the anti-MYC antibody. Experiments have been repeated four times.

To examine if the DOC1R protein was present in mouse oocytes, an affinity-purified anti-DOC1R antibody was produced and tested on a DOC1R protein produced in bacteria. A specific band of apparent molecular weight of 28 kDa is recognized with the antibody (Fig. 2B, lane 4) that disappears when the antibody is pre-incubated with the immunogenic peptide (Fig. 2B, lane 3). As the expected molecular weight of the tagged-DOC1R is ~19 kDa, the conformation of the protein must therefore modify its migration in SDS PAGE (see below). The antibody recognizes one band of about 28 kDa in mature mouse oocytes (Fig. 2B, lane 2) that also disappears when the antibody is pre-incubated with the immunogenic peptide (Fig. 2B, lane 1). The antibody seems highly specific for DOC1R, as it does not recognize any other protein on the immunoblot. As one mouse oocyte contains only 23 ng of total protein, this certainly explains why the antibody recognizes only a very faint band in mouse oocyte extracts (200 oocytes).

Therefore these experiments show that both DOC1R mRNA and protein are expressed in mouse oocytes.

DOC1R is regulated by phosphorylation during meiotic maturation of mouse oocytes

Because it required 200 oocytes to see a faint band corresponding to DOC1R on immunoblot, we followed the behavior of an overexpressed DOC1R protein during meiotic maturation after microinjection of RNA encoding MYC-DOC1R into immature oocytes. Samples of microinjected oocytes were collected at different times during meiosis

resumption. In contrast to MYC-MISS protein, the MYC-DOC1R protein accumulates in GV oocytes and at all stages of meiotic maturation (Fig. 3A) (Lefebvre et al., 2002). The apparent increase in MYC-DOC1R protein amount during meiosis is due to the progressive translation of injected RNA. In immature oocytes, the protein migrates to an apparent molecular weight of 26 kDa (Fig. 3A, lane 1). By contrast, during meiosis resumption the MYC-DOC1R protein up-shifts, migrating more slowly at about 30 kDa, suggesting that it is regulated by post-translational modifications.

We tested if these modifications could be due to phosphorylation. Immature oocytes in GV were injected with RNA encoding the MYC-DOC1R protein and collected in metaphase II. Half of the sample was treated with λ-phosphatase and the other half served as a control. As shown in Fig. 3B, the phosphatase treatment induces a down-shift of MYC-DOC1R electrophoretic mobility (compare lanes 1 and 2). This result indicates that DOC1R effectively undergoes phosphorylation during meiosis resumption.

The down-shift is not complete, suggesting that the treatment with λ-phosphatase was not totally efficient, or that the protein undergoes post-translational modifications other than phosphorylation.

Both cyclin B/CDC2 and MAPK phosphorylate DOC1R

As MYC-DOC1R undergoes post-translational modifications in oocytes collected one hour after GVBD, when MPF is active and MAPK inactive (Verlhac et al., 1994), we tested the ability of both kinases to phosphorylate DOC1R in vitro. For that, we incubated either purified cyclin B/CDC2 or active ERK2 in the presence of [γ-^{32}P]-ATP and purified DOC1R protein. As shown on the autoradiograph, both purified cyclin B/CDC2 and active MAPK are able to in vitro phosphorylate the DOC1R protein (Fig. 4A, lanes 1 and 4) to levels close to the histone H1 phosphorylation (Fig. 4A, lanes 2 and 5). This is consistent with the prediction of one MAPK phosphorylation site and one cyclin/CDK-binding site (Shintani et al., 2000) in the DOC1R protein sequence.

We took advantage of the *Mos*$^{-/-}$ mice, which do not activate MAPK (Colledge et al., 1994; Hashimoto et al., 1994; Verlhac et al., 1996), to show that MYC-DOC1R is an in vivo substrate of the MOS/…/MAPK pathway. As shown in Fig. 4B, the SDS PAGE migration profile of MYC-DOC1R from Mos$^{-/-}$ oocytes taken 12 hours after GVBD is completely different from wild-type oocytes and is characterized by three different bands from 26 to 30 kDa (compare lanes 1 and 2). The molecular weight of the lower band is the same as the DOC1R protein present in immature oocytes and the upper band migrates at an apparent molecular weight identical to the DOC1R protein present in mature wild-type oocytes.

To check whether the upper migrating forms of MYC-DOC1R present both in wild-type and *Mos*$^{-/-}$ oocytes share the same post-translational modifications, we performed 2D gel analysis. We observed that the upper band from wild-type oocytes resolved into two major isoforms (Fig. 4C, top panel). To position the different isoforms of MYC-DOC1R from one sample to the other, we used Ezrin as an internal control (not shown) (Louvet-Vallee et al., 2001). In metaphase II *Mos*$^{-/-}$ oocytes, the isoforms corresponding to the upper migrating band were shifted towards the basic pole (OH-, Fig. 4C, bottom panel). These results show first that the band migrating in 1D at an apparent molecular weight of 30 kDa corresponds to different isoforms of DOC1R. Second these results show that MYC-DOC1R is less phosphorylated in *Mos*$^{-/-}$ oocytes, as phosphorylations confer negative charges to proteins. These experiments suggest that the protein is effectively phosphorylated by the MOS/…/MAPK pathway and that other kinases are also responsible for DOC1R phosphorylation.

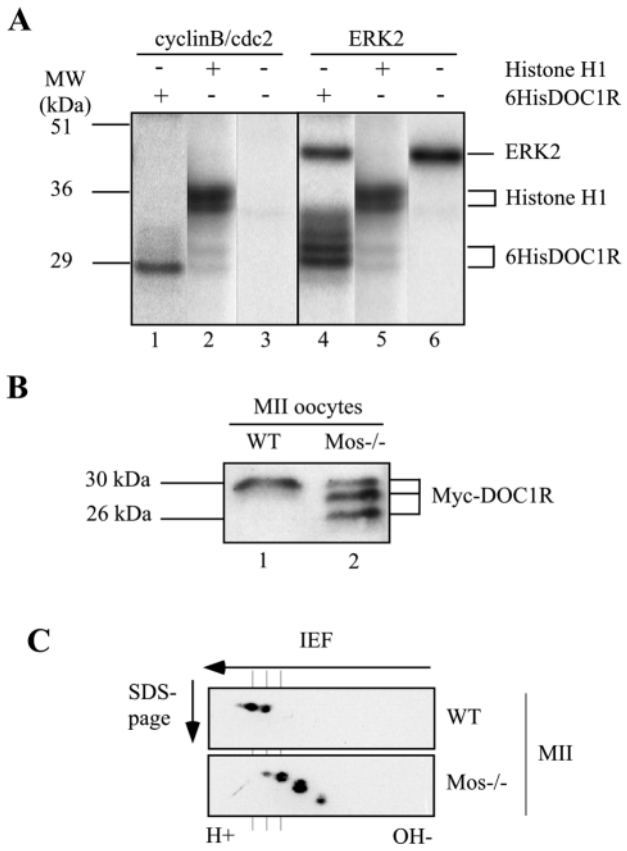


Fig. 4. cyclin B/CDC2 and MAP kinase phosphorylate DOC1R. (A) Purified cyclin B/CDC2 and MAPK phosphorylate DOC1R in vitro. Purified cyclin B/CDC2 (lanes 1, 2 and 3) and recombinant active rat ERK2 (lanes 4, 5 and 6) were incubated with (+) or without (–) 6His-DOC1R or Histone H1 (lanes 2 and 5) in the presence of [γ-^{32}P]-ATP. The [^{32}P] incorporation was detected by autoradiography. This experiment has been repeated twice. (B) The MOS/…/MAPK pathway phosphorylates DOC1R. MYC-DOC1R-injected oocytes from wild-type (lane 1) or *Mos*$^{-/-}$ (lane 2) mice were cultured for 12 hours after GVBD and collected. This experiment has been repeated three times. (C) MYC-DOC1R expression after microinjection of RNA encoding MYC-DOC1R into immature wild-type or *Mos*$^{-/-}$ oocytes. Forty wild-type oocytes cultured for 12 hours after GVBD (top panel) or 40 *Mos*$^{-/-}$ oocytes cultured for 12 hours (bottom panel) after GVBD were collected and analysed by 2D gel electrophoresis. The migration of MYC-DOC1R is shifted towards the acidic pole (H+) in wild-type oocytes compared with its migration in *Mos*$^{-/-}$ oocytes. To position the different MYC-DOC1R isoforms from one sample to the other, we re-probed all the blots using Ezrin as an internal control (Louvet-Vallee et al., 2001). This experiment has been repeated three times. Samples in B and C were analyzed by immunoblotting using an anti-MYC antibody.

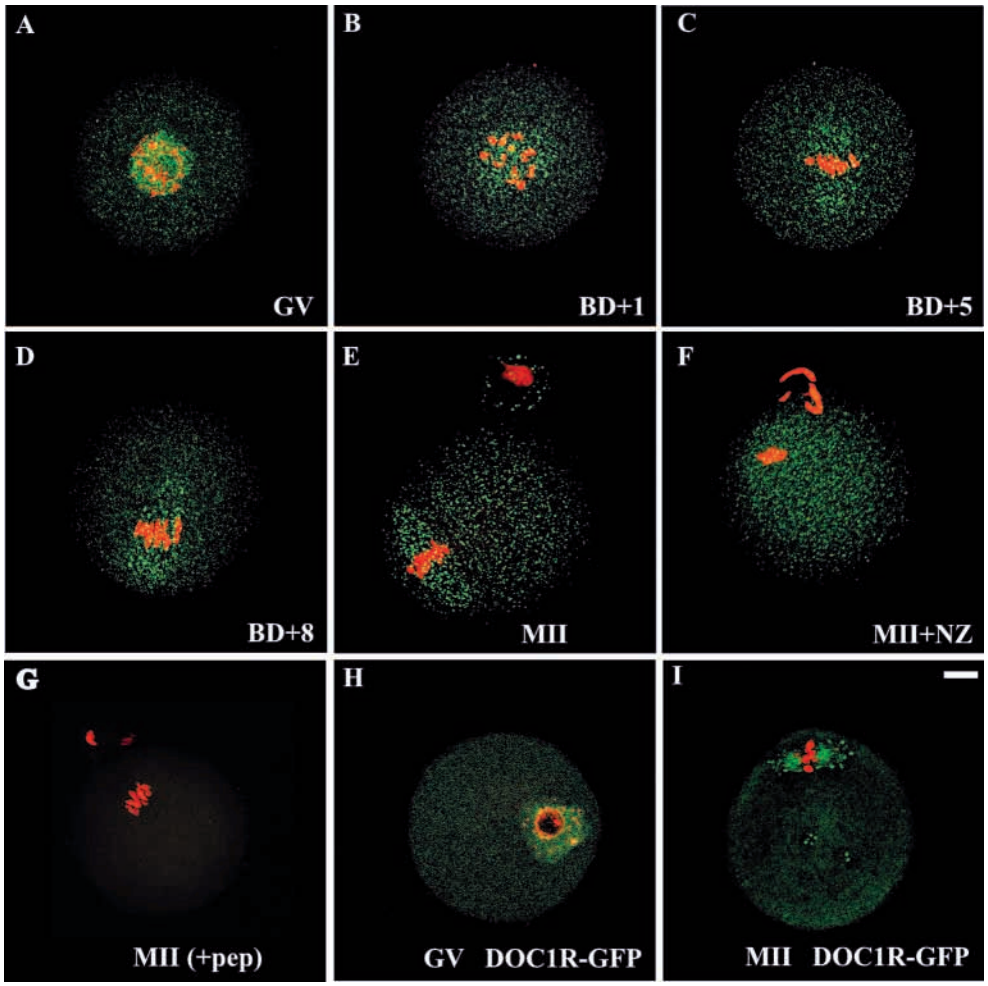


Fig. 5. DOC1R localizes to microtubules during meiotic maturation. (A-F) Localization of the endogenous DOC1R protein. Groups of 20 oocytes at different stages of meiotic maturation were fixed with formaldehyde and further stained with the anti-DOC1R antibody (A-F) or with the antibody blocked with 30 μM of immunogenic peptide (G). (H,I) Oocytes microinjected with DOC1R-GFP encoding RNA and collected in GV (H) or MII (I). All oocytes were analyzed by confocal microscopy using identical settings. (A) Immature oocyte in GV, (B) oocyte collected 1 hour after GVBD, (C) 5 hours after GVBD, (D) 8 hours after GVBD, (E,G-I) 14 hours after GVBD in metaphase II (MII), (F) cultured for 13 hours after GVBD then treated with nocodazole for 1 hour (MII+NZ). The DOC1R staining or DOC1R-GFP localization appear in green and chromosomes in red. Two-hundred and fifty oocytes were scored for this experiment. Scale bar: 10 μm.

DOC1R is present at all stages of meiotic maturation and localizes on metaphase spindles

We followed the endogenous DOC1R localization during meiotic maturation. We show that DOC1R is present in immature oocytes (Fig. 5A) and during all stages of meiotic maturation (Fig. 5B-E), confirming the immunoblotting analysis of the exogenous MYC-DOC1R protein. DOC1R accumulates in immature oocytes in the nucleus (Fig. 5A), as previously described in interphasic human cells (Zhang et al., 1999). During the first meiotic division (Fig. 5B-D), DOC1R accumulates in the cytoplasm and localizes in dots in the vicinity of the chromosomes in a region enriched in microtubules. Microtubule re-organization has been well described during mouse oocyte maturation (Brunet et al., 1999); microtubules form early during the first division around the chromosomes and organize into a bipolar spindle about 2 hours after meiosis resumption. DOC1R localization follows microtubule organization during metaphase I. In metaphase II (Fig. 5E), DOC1R also accumulates in the cytoplasm and on the spindle. To prove that the DOC1R protein is associated with microtubules, we treated metaphase II oocytes with nocodazole, which induces microtubule depolymerization (Fig. 5F). In these oocytes, the DOC1R protein was diffusely located in the cytoplasm, which proves that DOC1R associates specifically with spindle microtubules. As a control, we performed immunofluorescence after blocking the purified antibody with the immunogenic peptide and no staining was observed (Fig. 5G). Furthermore, like the endogenous DOC1R protein, a DOC1R-GFP fusion localizes in the germinal vesicle of immature oocytes (Fig. 5H) and on the metaphase II spindle (Fig. 5I).

The DOC1R depletion induces formation of asters of microtubules in the cytoplasm as well as at spindle poles of metaphase II oocytes

To investigate the role of DOC1R, we microinjected antisense RNA (asRNA), or double-stranded RNA (dsRNA) targeting the endogenous DOC1R mRNA. Oocytes were injected at the GV stage, collected at different stages of meiotic maturation and immunocytochemistry was performed to examine the chromosome and microtubule morphology (Fig. 6A-F). The injection of control dsRNA targeting the *Xenopus* Frizzled 7 mRNA did not affect meiotic maturation or spindle organization (Fig. 6B,K), with injected oocytes looking like non-injected ones in metaphase II (Fig. 6A,K). Typically, metaphase II-arrested oocytes present barrel-shaped spindles with no astral microtubules and no cytoplasmic asters of microtubules (Maro et al., 1985). When we injected asRNA or dsRNA against DOC1R mRNA, oocytes underwent their first meiotic division normally, extruded first polar bodies of normal size and with the same percentage as control oocytes (84.2±8.2% of polar body extrusion in the case of dsDOC1R

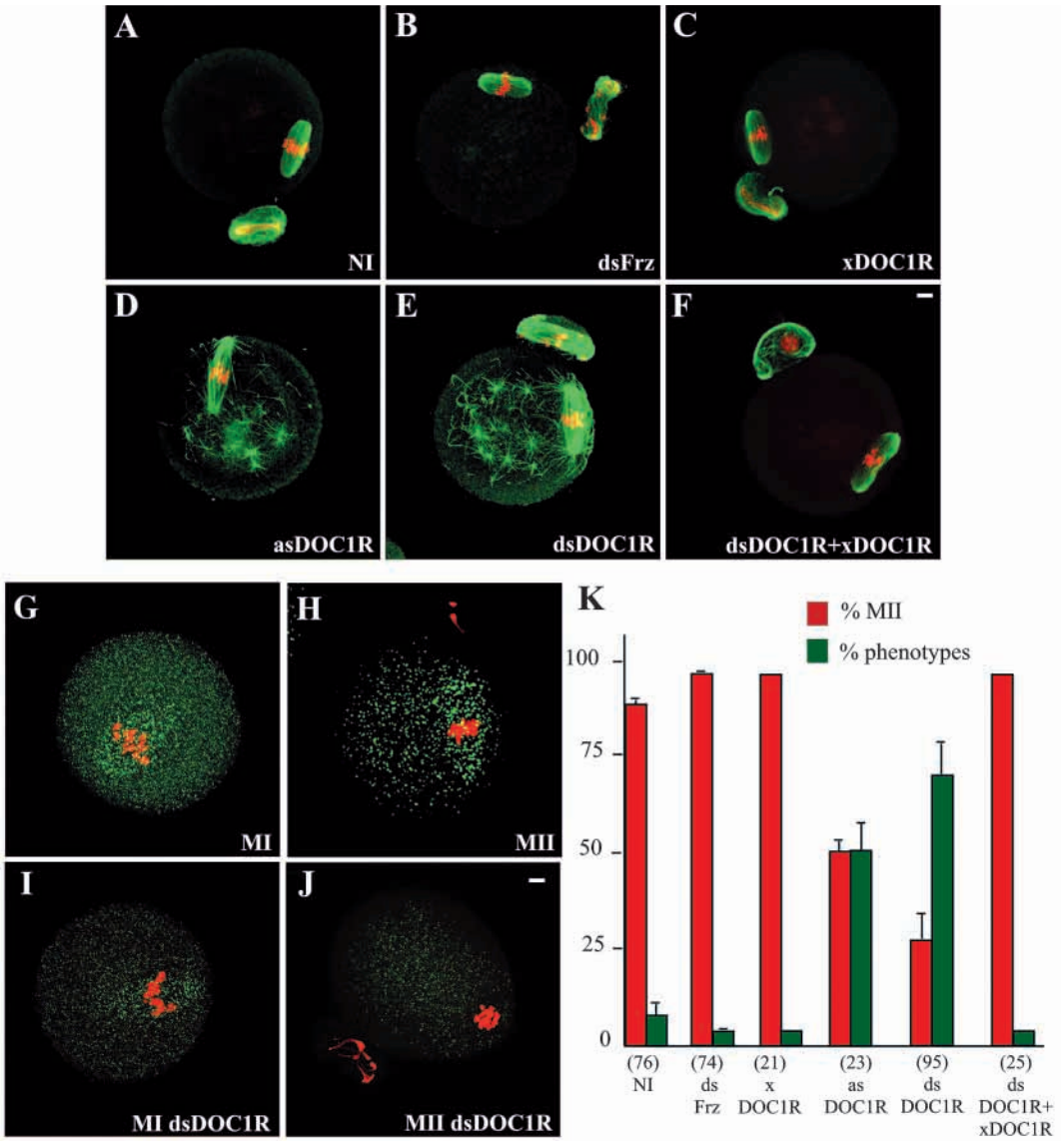


Fig. 6. DOC1R depletion induces formation of numerous microtubule asters at spindle poles as well as in the cytoplasm of MII oocytes. Immature oocytes were microinjected with antisense RNA (asDOC1R) or double-stranded RNA (dsDOC1R or dsFrz) directed against DOC1R or *Xenopus* Frizzled mRNA, or they were injected with RNA encoding *Xenopus* DOC1R with or without dsDOC1R, further cultured and collected at different stages of meiotic maturation. Oocytes were fixed with formaldehyde and analyzed by confocal microscopy. (A) Control non-injected oocyte (NI) collected in metaphase II. (B) Control oocyte injected with dsFrz collected in metaphase II. (C) Control oocyte injected with RNA encoding *Xenopus* DOC1R and collected in metaphase II. (D) Oocyte injected with asDOC1R collected in metaphase II. (E) Oocyte injected with dsDOC1R collected in metaphase II. (F) Oocyte injected with dsDOC1R and with RNA encoding *Xenopus* DOC1R, then collected in metaphase II. (G,H) Non-injected oocyte collected in metaphase I (MI, G) or metaphase II (MII, H). (I,J) Oocyte injected with the dsDOC1R collected in metaphase I (I) or II (J). For A-F, microtubules appear in green, for G,H, the endogenous DOC1R staining appears in green. For all images, chromosomes appear in red. Scale bars: 10 μm in A-F; 6 μm in G-J. (K) Statistics of the experiment described above. Percent MII: percentage of oocytes presenting a normal bipolar metaphase II spindle. Percent phenotypes: percentage of oocytes presenting spindle defects and numerous asters in the cytoplasm. The numbers in brackets correspond to the total number of oocytes analyzed. These experiments have been repeated between three to five times.

versus 84±12% in the control oocytes), but were arrested in metaphase II with severe phenotypes: spindles showing astral microtubules and numerous cytoplasmic asters (Fig. 6D,E). The injection of the asRNA induced 50% of such phenotypes while the injection of dsRNA was more efficient and induced 70% of elongated spindles (Fig. 6K).

To determine the efficiency of our dsRNA, we checked the presence of the endogenous DOC1R in the injected oocytes. The injection of dsRNA against DOC1R induces a loss of DOC1R localization on the MII spindle but not on the metaphase I spindles (Fig. 6 compare G with I and H with J).

To prove that the phenotype we observed was specific, we injected the dsRNA together with RNA encoding the *Xenopus* DOC1R protein. Despite the amino acid conservation between the murine and the *Xenopus* DOC1R proteins, the *Xenopus* cDNA diverges from the mouse, and thus is corresponding

cDNA cannot be targeted by the dsRNA. As shown on Fig. 6F,K, the *Xenopus* protein complements the microtubule defect induced by the dsRNA. The injection of the RNA coding the *Xenopus* protein has no effect by itself on microtubule organization (Fig. 6C). So the cytoplasmic asters as well as nucleation of microtubules from the spindle poles is solely due to DOC1R depletion.

Altogether, our experiments demonstrate that DOC1R regulates microtubule organization at least in metaphase II (see Discussion). The depletion of DOC1R induces severe damage to the microtubule cytoskeleton, which may compromise chromosome segregation after fertilization and hence compromise further embryo development.

Discussion

DOC1R regulation during meiotic maturation of mouse oocytes

DOC1R was isolated in the same screen that identified MISS, a protein required for metaphase II spindle stability. We show here that DOC1R is a MAPK partner as it interacts in a two-hybrid approach and co-immunoprecipitates with endogenous MAPK from *Xenopus* oocyte extracts.

DOC1R has a consensus site for MAPK phosphorylation, a potential CDK2 binding site and a potential cyclin/CDK binding site (Shintani et al., 2000). We show that DOC1R is expressed at all stages of meiotic maturation and that it is regulated by multiple phosphorylations. First, cyclin B/CDC2 is able to phosphorylate DOC1R in vitro, which is consistent with the presence of one potential cyclin/CDK binding site in its coding sequence. Furthermore, DOC1R becomes phosphorylated early in metaphase I, when MPF is active but not MAPK. Second, MAPK is able to phosphorylate DOC1R in vitro and in vivo, in agreement with the presence of one consensus site for MAPK phosphorylation in its sequence.

By 2D gel analysis, we show that the post-translational modifications that affect DOC1R during meiotic maturation are quite complex. Some modifications can be attributed to MPF activation, some to the MOS/…/MAPK pathway and it cannot be excluded that DOC1R is also modified by post-translational modifications other than phosphorylations. The characterization of DOC1R modifications by phosphorylation will be the object of further studies.

DOC1R function during meiotic maturation of mouse oocytes

Endogenous DOC1R is localized in dots on microtubules during all stages of meiotic maturation in particular on metaphase I and II spindles. These dots could reflect either an accumulation of DOC1R into vesicular structures associated with microtubules or macromolecular complexes containing DOC1R multimers.

Consistent with DOC1R association with microtubules, its depletion leads to drastic phenotypes in metaphase II arrested oocytes: elongated spindles enriched in astral microtubules and numerous asters of microtubules in the cytoplasm. The phenotype is specific to DOC1R depletion (1) because it is observed only when the endogenous protein can no longer be detected on the metaphase II spindle and (2) because overexpression of the *Xenopus* protein complements the defects observed after dsRNA injection.

The phenotype can be explained by extensive microtubule polymerization from MTOCs (microtubule organizing centers) that in mouse oocytes are present at spindle poles as well as foci in the cytoplasm (Maro et al., 1985). It can be interpreted as a reduced ability of the chromatin to stabilize microtubules in its vicinity. Normally, meiotic spindles are devoid of astral microtubules and cytoplasmic MTOCs do not form microtubule asters. The depletion of DOC1R promotes microtubule nucleation and/or elongation. This suggests that DOC1R normally increases microtubule dynamics in metaphase II arrested oocytes. We cannot exclude that DOC1R has a similar function in metaphase I, because we could not completely deplete the endogenous pool of DOC1R during the first meiotic division. The absence of a detectable phenotype in metaphase I could be explained by a low turnover of the protein and therefore a lack of full efficiency of the dsRNA during metaphase I.

DOC1R localization and the phenotype observed after its depletion are consistent with the potential tumor suppressor role of the DOC1R gene (Zhang et al., 1999). Indeed, human tumors are characterized by chromosomal instability primary resulting from spindle organization defects (Saunders et al., 2000).

A new vision of the metaphase II arrest of vertebrate oocytes

As for MISS, we could not find obvious invertebrate homologues of DOC1R. However, the protein sequence of DOC1R has been highly conserved from *Xenopus laevis* to human. Moreover, we show here that the *Xenopus* protein can functionally complement the mouse DOC1R. So we believe that both DOC1R and MISS mediate important functions specific to vertebrate species.

The meiotic metaphase II spindle harbors an unusual location in the cell: it is closely associated with the cortex, which is enriched in actin microfilaments. This association allows spindle rotation at fertilization, a prerequisite to second polar body extrusion that is essential for further embryo development. It is interesting that DOC1R is related to a coiled-coil region in a kinesin (KIF14) (for a review, see Miki et al., 2001) of unknown function that contains both a kinesin as well as a myosin domain. We can imagine that DOC1R somehow regulates interactions between spindle microtubules and microfilaments of the cortex that are necessary for maintaining proper MII spindle organization.

The discovery of new MAPK substrates, such as DOC1R and MISS, involved in the regulation of microtubule organization, is of crucial importance for our understanding of the processes controlling the metaphase II arrest of vertebrate oocytes. It suggests that the MOS/…/MAPK pathway not only controls CSF arrest by maintaining a high MPF activity through $p90^{rsk}$ activity (Bhatt and Ferrell, 1999; Gross et al., 1999; Gross et al., 2000) but also ensures that the spindle is properly organized during the arrest for the success of fertilization. Our findings are consistent with the unpublished data showing that the maintenance of bipolar spindles assembled in Xenopus egg extracts requires MAPK activity, but not $p90^{rsk}$ activity (Horne et al., 2003).

In conclusion, we have discovered two proteins that seem essential for mouse embryo development as their absence results in severe damage to the microtubule network of metaphase II mouse oocytes. Indeed *C. elegans mei-1* and

mei-2 mutants (proteins that regulate microtubule dynamics in meiosis and induce severing of microtubules to produce small meiotic spindles) extrude large polar bodies and show aneuploidy (Srayko et al., 2000).

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