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(54) Title: METHOD FOR THE IN VITRO DIAGNOSTIC OF INFERTILITY

(57) Abstract: The present invention relates to a method for the *in vitro* diagnostic of infertility of a eukaryotic organism, comprising the detection of the presence or the absence of Cdc6 protein or of Cdc6 mRNA in germinal cells of said eukaryotic organism, at a given maturation stage of said germinal cells.

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METHOD FOR THE IN VITRO DIAGNOSTIC OF INFERTILITY

The present invention relates to a method for the *in vitro* diagnostic of infertility of a eukaryotic organism.

Infertility, notably female infertility, arises from various causes. In women the main causes are pelvic inflammatory diseases, endometriosis, hypothalamic-pituitary disorders, polycystic ovarian syndrome, ovarian cysts, premature ovarian failure, luteal phase defect, or benign uterine fibroids.

Medical investigations to diagnose infertility and determine its etiology notably rely on *in vitro* tests, such as hormonal dosages, uterine biopsies, tests for autoimmune diseases, genetic tests, but also on imaging, with techniques such as echography or magnetic resonance imaging; or on physical investigations, such as hysteroscopy or hysterosalpingography.

However, about 15 to 20 % of female infertilities remain unexplained and thus untreated.

Cdc6 is a protein which is essential for the initiation of DNA replication (Kelly *et al.*, *Annu. Rev. Biochem.* (2000) **69**:829-880 ; Lei & Tye, *J. Cell Sci.* (2001) **114**:1447-1454). In conjunction with Cdct1 and the origin of replication complex (ORC) it is responsible for the loading of the mini-chromosome maintenance (MCM) complex onto the chromatin to form a pre-replication complex. This step establishes the license to replicate DNA, enabling the association of a replication complex to chromatin.

Oocyte maturation to give an unfertilized egg is a specialized stage of the meiotic cell cycle. In most species, oocytes are arrested at the prophase stage of the first meiotic division; they are not fertilizable and have lost the ability to replicate DNA. Hormone stimuli induce resumption of meiosis, with completion of meiosis I and arrest in meiosis II at the metaphase stage in vertebrates. At this stage, the mature oocyte can be fertilized; it has regained the ability to replicate DNA, and embryonic development can initiate.

The present invention provides a new method to detect a newly identified cause of sterility.

The invention relates in particular to a method for the *in vitro* diagnostic of infertility of a eukaryotic organism.

Another aspect of the invention relates to a pharmaceutical composition useful for curing infertility.

A further aspect of the invention relates to the use of a protein for the manufacture of a medicament for the treatment of infertilities.

Still another aspect of the invention relates to the use of a compound inhibiting a protein activity for the manufacture of a contraceptive.

The present invention relates to a method for the *in vitro* diagnostic of infertility of a eukaryotic organism, comprising the detection of the presence or the absence of Cdc6 protein or of Cdc6 mRNA in germinal cells of said eukaryotic organism, at a given maturation stage of said germinal cells.

According to a preferred embodiment, the invention more particularly relates to a method for the *in vitro* diagnostic of infertility of a female eukaryotic organism.

The expression "infertility" refers to the inability of said eukaryotic organism to sexually reproduce.

The expression "germinal cells" refers to cells which can yield gametes once differentiated or to the gametes themselves. In a female organism the gametes are called ovules.

The expression "ovule" relates to a fertilisable (unfertilized) egg.

The expression "maturation stage" refers to a particular step of the process of germinal cell differentiation, or meiotic maturation, to yield a gamete, and in particular to yield an egg from an oocyte.

In a female organism the germinal cells undergoing meiotic maturation are called maturing oocytes. Oocytes not having been through the first meiotic division are called primary oocytes, after having been through the first meiotic division they are called secondary oocytes. At the final stage of maturation the oocyte is called a mature oocyte. Once liberated from the ovary the mature oocyte is called an ovule.

In a female organism, infertility can in particular be tested by in vitro fertilization (IVF) on an ovule. The ovule, and thus the female organism, is said to be infertile if it does not undergo cell multiplication after the entry of the male gamete in the ovule under suitable conditions.

The invention relates in particular to a method as defined above, wherein the eukaryotic organism is a multicellular organism, in particular a plant, a fungus, or an animal, such as a mammal, in particular a human.

The invention further relates to a method as defined above, wherein the presence of Cdc6 protein is detected in oocytes prior to germinal vesicle breakdown.

The expression "germinal vesicle" refers to the nucleus of a non-mature oocyte.

At a particular stage of oocyte meiotic maturation, prior to metaphase I and after prophase I, the nucleus is fragmented into vesicles, it is said to undergo germinal vesicle breakdown (GVBD).

The expression "oocytes prior to germinal vesicle breakdown" refers to oocytes which have not yet been through the stage of germinal vesicle breakdown.

Oocytes are said to not to have been through GVBD if a nucleus is visible by microscopic observations for instance.

The presence of Cdc6 in oocytes prior to GVBD can be a cause of infertility. The presence of Cdc6 enables oocytes to undertake DNA replication, which may cause unwanted parthenogenetic development of the oocytes.

The invention also relates to the abovementioned method, wherein the absence of Cdc6 protein is detected in oocytes having undergone germinal vesicle breakdown, or in polar globules derived from oocytes having undergone germinal vesicle breakdown, or in ovules.

During the first division of meiotic maturation a primary oocyte divides to yield a secondary oocyte and a first polar body (or globule), each containing a set of duplicated chromosomes. During the second division of meiotic maturation a secondary oocyte divides to yield a mature oocyte and a second polar body (or globule), each containing a set of unduplicated chromosomes. Upon fertilization of the mature oocyte the first polar body duplicates.

The expression "polar globules derived from" refers to the first polar globules, duplicated or not, or to the second polar globule, which are yielded in the course of oocyte maturation.

The expression "having undergone germinal vesicle breakdown" refers to oocytes in which germinal vesicle breakdown has occurred.

Oocytes are said to have been through GVBD if no nucleus is visible by microscopic observations for instance.

The absence of Cdc6 in oocytes having undergone GVBD can be a cause of infertility. The absence of Cdc6 impairs the replication of DNA, and thus impairs embryogenic development.

According to another embodiment the present invention relates to a method as defined above, wherein the presence of Cdc6 protein is detected in oocytes prior to germinal vesicule breakdown, comprising a step of detection of the Cdc6 protein with an anti-Cdc6 protein polyclonal or monoclonal antibody.

Anti-Cdc6 protein antibodies can be obtained by methods well known to the man skilled in the art, in particular monoclonal antibodies can be obtained according to Köhler & Milstein *Nature* (1975) **256**:495-497.

Detection of the Cdc6 protein with an antibody can be achieved with several immunological methods well known to the man skilled in the art, such as Western blotting, immunocytochemistry, enzyme linked immnuoassay, immunofluorescence or mass spectrometry after proteolytic digestion.

The present invention also relates to a method as defined above, wherein the absence of Cdc6 protein is detected in oocytes having undergone germinal vesicle breakdown, or in polar globules derived from oocytes having undergone germinal vesicle breakdown, or in ovules, comprising a step of detection of the Cdc6 protein with an anti-Cdc6 protein polyclonal or monoclonal antibody.

The presence or the absence of the Cdc6 protein can be detected in oocytes prior to GVBD, having undergone GVBD, or in ovules, with methods well known to the man skilled in the art. Those methods comprise in particular Western blotting, immunocytochemistry, enzyme linked immnuoassay, immunofluorescence or mass spectrometry.

The present invention further relates to a method as defined above, comprising the following steps:

- sampling a polar globule from an egg, fertilized or not;
- contacting the sampled polar globule or extracts thereof with an anti-Cdc6 polyclonal or monoclonal antibody,
- detecting the presence or the absence of complexes between the Cdc6 protein and the anti-Cdc6 polyclonal or monoclonal antibody.

Advantageously, the sampling can be performed during an *in vitro* fecundation (IVF) procedure of an ovule by spermatozoids, or during intracytoplasmic sperm injection procedure (ICSI) into an ovule, before or after contacting sperm and the ovule.

Detection of the Cdc6 protein can be carried out in particular with the IDAT method. The expression IDAT refers to Immuno-Detection Amplified by T7 DNA polymerase, it is in particular described in Zhang *et al.*, *Proc. Natl. Acad. Sci. USA* (2001) **98**:5497-502.

According to another embodiment the present invention relates to a kit for carrying out the above mentioned process, comprising an anti-Cdc6 polyclonal or monoclonal antibody, optionally a medium in which the sampled polar globule is extracted for protein preparation, a medium in which the sampled polar globule, or the protein preparation extracted from said

polar globule, is contacted with said antibody, a reagent to detect the complex formed between Cdc6 and said antibody, and a control medium containing the Cdc6 protein.

Advantageously the antibody can be labelled, for instance with a fluorescent label, a radioelement, or an enzyme. Alternatively, the protein preparation can be labelled by fluorescent dyes such as Cy5™ or Cy3™ dyes (Research Organics, Cleveland, USA)

The present invention also relates to a pharmaceutical composition comprising as active substance a protein comprising or consisting of the Cdc6 protein or a part thereof, or a nucleic acid comprising or consisting of a nucleic acid encoding the Cdc6 protein or a part thereof, in association with a pharmaceutically acceptable carrier.

Pharmaceutically acceptable carriers notably comprise liposomes or Chariot™ carriers (Active Motif, Carlsbad, USA) for instance.

Advantageously the pharmaceutical composition comprises the Cdc6 protein as active substance.

The invention relates in particular to a pharmaceutical composition as defined above, wherein the nucleic acid is a RNA, in particular a polyadenylated RNA.

The expression "polyadenylated RNA" refers to a RNA which carries a 3' polyadenosine tail.

The invention more particularly relates to a pharmaceutical composition as defined above, wherein the Cdc6 protein comprises a sequence corresponding to the human Cdc6 protein sequence, said sequence corresponding in particular to SEQ ID NO: 2, or wherein the nucleic acid encoding the Cdc6 protein comprises a sequence corresponding to the human Cdc6 nucleic acid sequence, said sequence corresponding in particular to SEQ ID NO: 1.

The expression human Cdc6 refers to a Cdc6 protein or a Cdc6 encoding nucleic acid, the sequences of which are identical to the sequences of Cdc6 proteins or Cdc6 encoding nucleic acids which can be found in natural human cells.

Human Cdc6 is in particular described in Williams *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* (1997) **94**:142-147.

According to another embodiment the present invention relates to the use of a protein comprising or consisting of the Cdc6 protein or a part thereof, or of a nucleic acid comprising or consisting of a nucleic acid encoding the Cdc6 protein or a part thereof, for the manufacture of a medicament for the treatment of infertilities.

The Cdc6 protein or a nucleic acid encoding said protein, can be administered to an unfertile ovocyte or ovule.

According to a preferred embodiment the delivery of the Cdc6 protein or the Cdc6 mRNA is carried out by cell microinjection.

According to another preferred embodiment in the case of ICSI, sperm injection into the ovule is accompanied by Cdc6 protein or Cdc6 protein encoding nucleic acid administration, in particular injection, into the ovule.

The invention relates in particular to the use as defined above, wherein the nucleic acid is a RNA, in particular a polyadenylated RNA.

The invention further relates to the use as defined above, wherein the Cdc6 protein comprises a sequence corresponding to the human Cdc6 protein sequence, said sequence corresponding in particular to SEQ ID NO: 2, or wherein the nucleic acid encoding the Cdc6 protein comprises a sequence corresponding to the human Cdc6 nucleic acid sequence, said sequence corresponding in particular to SEQ ID NO: 1.

According to another embodiment the present invention relates to the use of a compound inhibiting Cdc6 protein activity, such as a pharmacological compound, an antisense polynucleotide directed against the mRNA encoding the Cdc6 protein, a siRNA inhibiting Cdc6 protein expression, an anti-Cdc6 protein antibody or fragments thereof, such as a Fab fragment or a scFv fragment, for the manufacture of a contraceptive.

The expression "antisense polynucleotide" refers to a polynucleotide the sequence of which is essentially complementary to a target sequence, said target sequence being in particular located within the sequence of Cdc6 mRNA

The expression "siRNA" relates to a double stranded RNA which can silence the expression of specific genes, advantageously the sequence of said siRNA comprises or consists of at least a part of a sequence of a nucleic acid encoding the Cdc6 protein.

The expression "Fab fragment" relates to antibody fragments which can be obtained by papaine digestion of antibodies.

The expression "scFv fragment" refers to a single chain Fv fragment, it is usually recombinant.

The expression "contraceptive" refers to a compound which blocks sexual reproduction.

The invention more particularly relates to the use as defined above, wherein the Cdc6 protein corresponds to a human Cdc6 protein.

The present invention also relates to a phytosanitary composition comprising as active substance a protein comprising or consisting of the Cdc6 protein or a part thereof, or a nucleic acid comprising or consisting of a nucleic acid encoding the Cdc6 protein or a part thereof, or a compound inhibiting Cdc6 activity, such as an antisense nucleotide directed against the

mRNA encoding the Cdc6 protein, a siRNA inhibiting Cdc6 protein expression, an anti-Cdc6 protein antibody or fragments thereof, such as a Fab fragment or a scFv fragment.

The expression "phytosanitary" composition relates to a composition useful for the treatment of plants.

The invention relates in particular to the above defined phytosanitary composition, wherein the Cdc6 protein corresponds to a plant Cdc6 protein.

The expression "plant Cdc6" refers to a Cdc6 protein or a Cdc6 encoding nucleic acid, the sequences of which are identical to the sequences of Cdc6 proteins or Cdc6 encoding nucleic acids which can be found in natural plant cells

According to another embodiment the present invention relates to the use of a protein comprising or consisting of the Cdc6 protein or a part thereof, or of a nucleic acid comprising or consisting of a nucleic acid encoding the Cdc6 protein or a part thereof, or of a compound inhibiting Cdc6 protein activity, such as an antisense polynucleotide directed against the mRNA encoding the Cdc6 protein, a siRNA inhibiting Cdc6 protein expression, an anti-Cdc6 protein antibody or fragments thereof, such as a Fab fragment or a scFv fragment, for the treatment of a plant or parts thereof, or for the inhibition of plant reproduction.

The invention relates in particular to the above defined use of a protein comprising or consisting of the Cdc6 protein or a part thereof, or a nucleic acid comprising or consisting of a nucleic acid encoding the Cdc6 protein or a part thereof, for the treatment of plant infertility.

The invention more particularly relates to the above defined use of a compound inhibiting Cdc6 protein activity, such as an antisense polynucleotide directed against the mRNA encoding the Cdc6 protein, a siRNA inhibiting Cdc6 protein expression, an anti-Cdc6 protein antibody or fragments thereof, such as a Fab fragment or a scFv fragment, as an herbicide.

The expression "herbicide" relates to a compound which inhibits plant growth and/or reproduction, in particular sexual reproduction.

The invention further relates to the use as defined above, wherein the Cdc6 protein corresponds to a plant Cdc6 protein.

DESCRIPTION OF THE FIGURES

Figure 1

Figure 1 represents immunoblots, revealed with antibodies directed against *Xenopus* CDC6, of a SDS-PAGE of total, cytoplasmic and germinal vesicle (GV) fractions of *Xenopus*

oocytes, and of CSF (ie containing the cytostatic factor) and interphasic extracts of *Xenopus* eggs. The bands approximately migrating to 60 kD correspond to Cdc6.

Figure 2

Figure 2 represents the immunoblotting of a SDS-PAGE of extracts from *Xenopus* oocytes derived from stage 6 oocytes induced to mature by progesterone (PG). GVBD occured 2 h 45 min after progesterone addition (GVBD). Extracts were collected 0, 1, 2, 3, 4.5, 6.5 or 7.5 hours after the addition of progesterone. Some oocytes were also treated with cycloheximide either 30 min before GVBD (cyclo before GVBD), or 1 hour after GVBD (cyclo after GVBD), and collected at the Metaphase I / Metaphase II transition (2h 30 min after GVBD). The immunoblot was revealed with antibodies directed against CDC6, ORC2, MCM4, MCM3 or CDC45.

Figure 3

Figure 3 represents a Northern blot of extracts from *Xenopus* oocytes derived from stage 6 oocytes induced to mature by progesterone (PG) and revealed by a labelled anti-Cdc6 mRNA probe. GVBD occured 2 h 45 min after progesterone addition (GVBD). Extracts were collected 0, 1, 2, 3, 4.5, 6.5 or 7.5 hours after the addition of progesterone. Some oocytes were also treated with cycloheximide either 30 min before GVBD (cyclo before GVBD), or 1 hour after GVBD (cyclo after GVBD), and collected at the Metaphase I / Metaphase II transition (2h 30 min after GVBD). Some oocytes were also treated with Actinomycin D.

Figure 4

Figure 4 represents the immunoblotting, revealed with antibodies directed against *Xenopus* Cdc6, of a SDS-PAGE of extracts from stage 6 oocytes, or stage 6 oocytes injected with 50 ng of 25-mer sense Cdc6 oligonucleotide, 50 ng of 25-mer antisense Cdc6 oligonucleotide, or or water (mock), and induced to mature by PG. Matured oocytes were collected 12 hours after PG stimulation

Figure 5A, Figure 5B and Figure 5C

Figure 5A represents the DNA synthesis ability, analyzed by agarose gel electrophoresis, of *Xenopus* oocytes injected with 25 nl of a 2 mg/ml solution of purified Cdc6 sense oligonucleotides (lane 1), the corresponding antisense oligonucleotides (lanes 2, 3), or water (lane 4) and induced to mature. Rescue experiments were assayed by a second injection

performed soon after GVBD that contained 0.25 μCi α32-PdCTP (lanes 1-4) and either boiled-inactivated (lane 2) or intact purified Cdc6 (lane 3). The arrow on the left marks the migration of the bands corresponding to the newly synthesized DNA.

Figure 5B represents the DNA synthesis ability, analyzed by agarose gel electrophoresis, of *Xenopus* oocytes injected with water (lane 1), antisense oligonucleotides (lanes 2, 3) or sense oligonucleotides (lane 4) and fully matured during twelve hours. DNA replication was analyzed by injection of 0.25 μCi α32P-dCTP in absence (lanes 1, 2, 4) or presence of recombinant Cdc6 protein (lane 3). The arrow on the left marks the migration of the bands corresponding to the newly synthesized DNA.

Figure 5C represents the DNA synthesis ability analyzed by agarose gel electrophoresis of *Xenopus* oocytes induced to mature and treated with 250 μg/ml cycloheximide added 30 min before GVBD (lane 2) or 1 hour after GVBD (lane 4). Maturing oocytes were injected soon after GVBD with 0.25 μCi of α32P-dCTP without (lanes 1, 4) or with active (lane 2) or inactivated (lane 3) recombinant Cdc6 protein. The arrow on the left marks the migration of the bands corresponding to the newly synthesized DNA

Figure 6A, Figure 6B and Figure 6C

Figure 6A represents the DNA synthesis ability analyzed by agarose gel electrophoresis of stage 6 *Xenopus* oocytes induced to mature by PG, either non treated (-cyclo) or treated with 250 μg/ml cycloheximide 30 min before GVBD (cyclo –30 min), or 1 hour after GVBD (cyclo +1h). Oocytes extracts were collected at the metaphaseI/metaphaseII (MI/MII) transition (2 hrs 30 min after GVBD). Matured oocytes were also collected 12 h after PG addition. Sperm nuclei were added to the extracts and DNA synthesis was followed by agarose gel electrophoresis, after 2 hours.

Figure 6B represents the percentage of DNA replication after 2 hours (vertical axis) for a metaphase I/II extract from maturing oocytes either treated by:

- cycloheximide before GVBD, and water (mock), Cdc6 protein (+ CDC6) or Cdc6 protein and aphidicolin (+ CDC6 Aphi), or
- cycloheximide after GVBD and water (mock) or aphidicolin (+ Aphi).

Figure 6C represents the percentage of DNA replication after 2 hours (vertical axis) for:

- a GVBD extract trated by water (mock), Cdc6 protein (+ CDC6) or Cdc6 protein and aphidicolin (+ CDC6 Aphi), or
- a metaphase I/II extracts treated by water (mock) or aphidicolin (+ Aphi).

Figure 7

Figure 7 represents an immunoblot of a SDS-PAGE of extracts from *Xenopus* or mouse oocytes in prophase I, metaphase I or metaphase II. The immunoblot was revealed with antibodies directed against *Xenopus* CDC6 and MCM2, or mouse CDC6 and MCM2.

Figure 8

Figure 8 represents an immunoblot of a SDS-PAGE of extracts from virgin female *Drosophila melanogaster* at stage 14 (lane 1), or activated oocytes (lane 2). The immunoblot was revealed with antibodies directed against Cdc6 (DmCDC6), MCM2 (DmMCM2), and tubulin (DmTub).

EXAMPLES

Example 1

Cdc6 confers competence to replicate in the unfertilized egg of *Xenopus laevis*

A. Materials and methods

1. Fractionation of oocytes

Defolliculated oocytes were prepared as described in Menu *et al.* in *Advances in Molecular Biology* (1999) Oxford University Press, Ed. J.D. Richter. Cytoplasmic and germinal vesicle fractions were obtained after manual enucleation either from intact oocytes or oocytes fixed by 2 % TCA to prevent possible leaks from the nucleus. Protein extracts were obtained by grinding oocytes in 100 mM KCl, 5 mM $MgCl_2$, 0.1 mM $CaCl_2$, 10 mM K/Hepes pH 7.7, 50 mM sucrose, 5 µg/ml leupeptin, 5 µg/ml pepstatin, 5 µg/ml aprotinin, 0.5 mM EGTA, 0.3 % X100 Triton, centrifuged 5 min at 8 000 g, and further analyzed by SDS-PAGE.

2. Oocytes extracts

Low Speed Extracts of maturing oocytes were performed at GVBD (germinal vesicle breakdown) (GVBD extract) or between MI/MII, 2h30 after GVBD (MI/MII extract). GVBD, judged by the appearance of a white spot at the animal pole, started at 2 hrs 45 – 3 hrs 30 min. Maturing oocytes extracts were performed as for egg extracts in eppendorf tubes (Menu *et al.* in *Advances in Molecular Biology* (1999) Oxford University Press, Ed. J.D. Richter). After removing the excess buffer, oocytes were centrifuged at 8000 g, 5 min at 4°C, and then 12000 g, 2 min at 4°C. The upper phase was collected and centrifuged again at 12000 g for 2 min at 4°C. Extracts were collected and supplemented with energy mix (Menu *et al.* in *Advances in Molecular Biology* (1999) Oxford University Press, Ed. J.D. Richter).

3. DNA replication assays

Oocytes were injected with 25 nl of 5 mCi/ml 32P-dCTP, collected 5 hours after GVBD, and subjected to DNA extraction. Samples with equal numbers of total counts were analyzed by 0.8 % agarose gel electrophoresis. DNA replication assays were also performed by quantification of α32P-dCTP incorporation in sperm nuclei used at 1000 nuclei/µl of extract. Analysis was both done by TCA precipitation and migration on 0.8 % Agarose (Menu *et al.* in *Advances in Molecular Biology* (1999) Oxford University Press, Ed. J.D. Richter).

Purified protein was added at 15 ng/µl of extract to rescue DNA replication activity. Interphasic extract (Int) and CSF extract (ie extracts containing the mitotic cytostatic factor) were prepared as described in Menu *et al.* in *Advances in Molecular Biology* (1999) Oxford University Press, Ed. J.D. Richter and Lemaître *et al.*, *J. Cell Biol.* (1998) **142**:1159-1166.

4. Northern blot analysis

Total oocyte RNA was extracted using a LiCl method (Taylor *et al.*, *EMBO J.* (1986) **5**:3563-3570). 10 µg of total RNA was submitted to electrophoresis in 1 % formaldehyde agarose gel and transferred to Amersham Hybond N+ membrane.

5. Intracellular injections of nucleotides and proteins

Stage 6 oocytes were injected with 25 nl of a 2 mg/ml solution of purified Cdc6 25 mers sense oligonucleotide (5'-TCCCCACCCAAGCAGTCTCGCAAAG-3') (SEQ ID NO: 3) or 25 mers of the corresponding antisense (5'-CTTTGCGAGACTGCTTGGGTGGGGA-3') (SEQ ID NO: 4) oligonucleotide, and induced to mature by addition of progesterone. Control oocytes were injected in parallel with water. For rescue experiments a 25 nl second injection containing 5 mCi/ml dCTP and 300 µg/ml purified Cdc6 protein was injected after GVBD or in 12 hours-matured oocyte. Maturation was followed by appearance of a clear white spot at the animal hemisphere as well as manual dissection to check for germinal vesicle breakdown.

6. Cdc6 protein and antibodies

Xenopus His6-Cdc6 protein was purified from recombinant baculovirus-infected insect cells. Its activity was monitored by depletion–rescue experiments as follows: 50 µl of low speed interphasic extract was depleted with Cdc6 specific polyclonal antibody, or mock-depleted with preimmune serum IgG, as previously described (Maiorano *et al.*, *Nature* (2000) **404**:622-625). Cdc6 purified protein was added to the depleted extract and DNA replication was then measured. The rabbit polyclonal anti-Cdc6 antibodies were obtained by 4 injections of Cdc6 protein.

B. Results

1. Detection of the Cdc6 protein

The analysis of replication initiation factors present in the oocyte revealed that Cdc6, a critical factor involved in the assembly of the MCM helicase complex at DNA replication origins (Kelly *et al.*, *Annu. Rev. Biochem.* (2000) **69**:829-880 ; Lei & Tye, *J. Cell Sci.* (2001) **114**:1447-1454), was undetectable in fully grown oocytes but present in mature oocytes or in activated eggs (**Figure 1**). This was in contrast to other initiation proteins analyzed that were present in the oocyte, either nearly exclusively in the nucleus (MCM subunits, RPA70, RPA34), or also detected in the cytoplasm (geminin, cdt1, cdc7, ORC, cdc45). Cdc6 electrophoretic migration shifts down in interphasic egg extract (**Figure 1**), in agreement with previous observations (Coleman *et al.*, *Cell* (1996) **87**:53-63).

Although Cdc6 was absent in the oocyte, a relatively large amount of the protein was detected in the unfertilized egg, 5 ng / unfertilized egg, an estimate comparable with the 3 ng-value previously reported (Coleman *et al.*, *Cell* (1996) **87**:53-63). As Cdc6 is an essential factor for replication licensing, the Inventors asked at what point during maturation it was accumulated. During maturation by progesterone (**Figure 2**), the Cdc6 protein becomes detectable soon after germinal vesicle breakdown (GVBD, e.g. 3 hrs after progesterone addition), when MCM4 is phosphorylated by MPF (Coué *et al.*, *EMBO J.* (1996) **15**:1085-1097 ; Pereverzeva *et al.*, *Mol. Cell Biol.* (2000) **20**:3667-3676). Inhibition of protein synthesis by cycloheximide added before GVBD, but not 1 hr afterwards, results in the absence of Cdc6 in the egg (**Figure 2**). These data show that Cdc6 protein is missing in the oocyte and starts to accumulate during maturation.

2. Detection of Cdc6 mRNA

Although Cdc6 protein is absent in oocytes, Northern blots show that Cdc6 mRNA was already stored as a maternal mRNA during oogenesis (**Figure 3**). In addition, a shift in electrophoretic mobility of the Cdc6 mRNA was detected soon after GVBD, consistent with a polyadenylation process during maturation (**Figure 3**). This shift is still detected if cycloheximide is added 1 hour after GVBD, but is extremely reduced if cycloheximide is added 30 min before GVBD, consistent with the respective absence and presence of Cdc6 protein in maturing oocyte (**Figure 2**). Actinomycin D, which prevents DNA synthesis, present during maturation did not inhibit this shift (**Figure 3**), suggesting that Cdc6 synthesis was regulated at the translational level, by polyadenylation of a maternal Cdc6 mRNA pool.

3. Cdc6 synthesis inhibition by injection of antisense oligonucleotides

In addition, Cdc6 protein accumulation in the egg is blocked by injection of antisense cdc6 oligonucleotides in the oocyte, before maturation, in contrast to oocytes injected with the corresponding sense oligonucleotides (**Figure 4**). These results suggest that synthesis of Cdc6 protein during oocyte maturation relies on translational control of Cdc6 mRNA stored in the oocyte.

4. Injection of recombinant Cdc6 in the egg

To address if Cdc6 was the missing replication factor, sufficient to confer the competence for DNA replication to the egg, the hypothesis according to which recombinant Cdc6 was sufficient to provide this competence, was tested. A recombinant Cdc6 produced from baculovirus-infected cells was purified and was shown to be active in rescuing DNA replication in *Xenopus* egg extracts that were Cdc6-depleted using a purified specific polyclonal Cdc6 antibody (see Materials and Methods section). The purified recombinant protein was also used in the *in vivo* rescue experiments described below.

5. Inhibition of replication by injection of antisense oligonucleotides and rescue by Cdc6 injection

The activity of the missing DNA synthesis inducer that is synthetized during maturation is normally repressed by MPF to prevent DNA replication between meiosisI/meiosisII (Furuno *et al.*, *EMBO J.* (1994) **13**:2399-2410). Addition of cycloheximide 1 hour after GVBD prevents reaccumulation of MPF and consequently induces unscheduled DNA replication between meiosisI/meiosisII (Furuno *et al.*, *EMBO J.* (1994) **13**:2399-2410). To address if this induced ability to replicate required Cdc6 synthesis during maturation, the prevention of unscheduled replication by Cdc6-mRNA depletion using a Cdc6 antisense oligonucleotide was first tested. **Figure 5A** shows that addition of cycloheximide one hour after GVBD induces DNA replication between meiosisI/meiosisII, as reported (Furuno *et al.*, *EMBO J.* (1994) **13**:2399-2410), but this induction is inhibited by Cdc6 antisense oligonucleotides. In addition, injection of recombinant Cdc6 (**Figure 5A**) rescues induction of unscheduled replication.

The inventors further asked if Cdc6 was sufficient to give competence to replicate in-vivo to the matured oocyte. *In vivo*, after maturation, parthenogenetic activation triggered by a micropipette injection of water for instance induces DNA replication (Gurdon *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* (1967) **58**:545-552 ; Hara et al., *Proc. Natl. Acad. Sci. U.S.A.* (1980)

77:462-466 ; Harland & Laskey, *Cell* (1980) **21**:761-771) as shown in **Figure 5B** (lane 1). Injection of Cdc6 antisense oligonucleotides in the oocyte before maturation inhibits DNA replication (**Figure 5B**, lane 2), but microinjection of Cdc6 protein after maturation is sufficient to rescue DNA replication (lane 3) with an efficiency similar to control oocytes treated with sense oligonucleotides (**Figure 5B**, lane 4).

The inventors concluded that Cdc6 is the missing factor synthesized during oocyte maturation sufficient to give (i) replication-induced ability between meiosisI/meiosisII, and (ii), competence to replicate in the unfertilized egg.

Previous work has shown that maturing oocytes can replicate DNA after meiosis I by cycloheximide treatment 60 min after GVBD, but not 30 min before GVBD (Furuno *et al.*, *EMBO J.* (1994) **13**:2399-2410). These findings were reproduced by the Inventors, confirming that competence for DNA replication *in vivo* is acquired shortly after GVBD, and is dependent on translation (**Figure 5C**, compare lane 1 and 4). In addition, the Inventors have shown show that microinjection of Cdc6 after translation inhibition 30 min before GVBD is sufficient to rescue induced-replication ability between meiosis I and meiosis II (**Figure 5C**, lanes 1, 2, 3).

6. *In vitro* observations

This set of *in vivo* observations could be reproduced *in vitro*, in oocyte extracts prepared between meiosisI/meiosisII (Blon *et al.*, *Cell* (1986) **47**:577-587 ; Menu *et al.* in *Advances in Molecular Biology* (1999) Oxford University Press, Ed. J.D. Richter, and Methods). Oocytes were matured *in vitro* and cycloheximide was added 30 min before or 60 min after GVBD. Extracts were made 2 hrs 30 min post-GVBD, to which sperm nuclei were added to follow DNA replication ability. If cycloheximide is added 60 min after GVBD, DNA replication occurs, as expected (**Figure 6A and 6B**), in a reaction entirely aphidicolin-sensitive (**Figure 6B**). If cycloheximide is added 30 min before GVBD, Cdc6 is not translated (**Figure 2B**), and extracts are incompetent to replicate (**Figure 6A and 6B**). However, addition of recombinant Cdc6 to the extract is sufficient to rescue replication, in an aphidicolin-sensitive reaction (**Figure 6B**). Moreover, the simple addition of recombinant Cdc6 to oocyte extracts made at GVBD is sufficient to confer DNA replication competence, with an efficiency close to that obtained in egg extracts (**Figure 6C**). These results further demonstrate that Cdc6 is the missing DNA replication factor starting to be translated at GVBD, necessary and sufficient to confer to the egg the ability to replicate embryonic DNA after fertilization, when the activity MPF is suppressed.

Example 2

CDC6 confers competence to replicate in the unfertilized egg of *Drosophila melanogaster*

Of the established model organisms, *Drosophila melanogaster* provides a particularly potent system to study whether in invertebrate organism Cdc6 appears during meiosis to give the competence to replicate DNA to the egg. Like many other animals, female *D. melanogaster* generate oocytes but store them, arrested and unactivated, in the ovary. After a period of growth, *Drosophila* oocytes arrest in metaphase of meiosis I, with egg coverings (a vitelline envelope and chorion). Ovulation releases these mature eggs from the ovary and triggers the process known as activation.

Although *D. melanogaster* mature virgin females spontaneously ovulate at a very low rate (~1 egg/day), nutritional supplementation causes increased germ cell proliferation resulting in increased ovulation rates (Drummond-Barbosa and Spradling, *Dev. Biol.* (2001) **231**:265-278).

The Inventors have compared activated eggs laid by a virgin female with stage 14 oocytes blocked at Metaphase I, collected directly from the ovaries. Both were treated to remove the vitelline envelope and chorion. The activation of the oocytes triggers the synthesis of Cdc6 that was absent from the stage 14 (metaphase I oocyte), while MCM2 was already present at stage 14 (**Figure 8**). Despite several differences in egg formation *D. melanogaster* accumulates Cdc6 betwen metaphase I and metaphase II, before fertilisation. Moreover, Cdc6 immunostaining during oocyte growth revealed a positive signal in the cytoplasm of nurse cells and cells covering the oocyte, while MCM2 was mostly nuclear, consistent with cell cycle regulation of Cdc6 function. Thus, in the invertebrate *D. melanogaster*, like in the vertebrate *X. laevis*, Cdc6 is absent from prophase I oocyte and translated between Metaphase I and Metaphase II to give to the egg the competence to replicate before fertilisation.

Example 3

CDC6 confers competence to replicate in the unfertilized egg of *Mus musculus*

Oocyte extracts were prepared as described above and analyzed by SDS-PAGE. Immunoblots were revealed with antibodies directed against *Xenopus laevis* Cdc6 and MCM2, as well as mouse Cdc6 and MCM2. Prophase I oocytes were induced to mature by addition of progesterone (PG). Extracts from 5 *X. laevis* maturing oocytes or 85 mice oocytes

were separated by SDS PAGE and examined by immunoblotting. The results are shown in Figure 7. Mouse Cdc6 is absent from prophase I oocyte and translated between Metaphase I and Metaphase II, which is compatible with its ability to confer competence to replicate to the egg before fertilization.

Thus mammalian Cdc6 appears to be a potent target for the development of new contraceptives. Besides, its essential role in the acquisition of the competence to replicate in the egg strongly suggests that it might be involved in several infertilities, which could be overcome by providing Cdc6 protein or Cdc6 mRNA to infertile eggs or oocytes.

Example 4

Detection of the presence or the absence of the CDC6 protein by immunocytochemistry methods

The following method is used for the detection of the Cdc6 protein, in particular for the detection of the human Cdc6 protein in germinal cells, ovocytes, ovules or polar bodies:

1) Ovulated oocytes or oocyte or polar bodies, for instance obtained before or after "*in vitro* fertilization" (IVF) or "intracytoplasmic sperm injection" (ICSI), are transferred to a sterile watch glass containing fixation buffer (20 mM Pipes, 1mM $MgCl_2$, 0.5 mM EGTA, 1 mM DTT, 1 µM Taxol, 0.1 % X100 triton, 2 % Formaldehyde) at 37°C for 30 minutes.
2) Transfer oocytes for three washes in 0.1 % Normal Goat Serum in PBS at 37°C for 5 minutes each.
3) Tranfer oocytes to 10% Normal Goat Serum for one hour (Oocytes can be stored in 10% Normal Goat Serum at 4)C prior to immunostaining).
4) Tranfer oocytes to anti-Cdc6 antibody diluted in 5% Normal goat serum and place them at 37°C for 1 hour.
5) Transfer oocytes through 3 washes of 10 % Normal Goat Serum for 5 minutes each.
6) Transfer oocytes to appropriate secondary antibody FITC-conjugated and place them at 37°C for 1 hour.
7) Transfer oocytes through three washes in 10 % Normal Goat Serum for 5 minutes each.
8) Transfer oocytes to Hoescht 33258 500ng/ml in PBS for 2 minutes to stain DNA.
9) Tranfer on a microscope slide and add a drop of mounting medium and cover with a glass coverslip.

10) The oocyte can be observed under a fluorescence microscope for Cdc6 immunostaining. A negative signal is indicative of an unfertilizable ovulated oocyte.

The detection of the presence or the absence of Cdc6 detected by this method provides the basis for an in vitro diagnostic method of infertilities linked to a deficiency in active Cdc6.

CLAIMS

1. A method for the *in vitro* diagnostic of infertility of a eukaryotic organism, comprising the detection of the presence or the absence of Cdc6 protein or of Cdc6 mRNA in germinal cells of said eukaryotic organism, at a given maturation stage of said germinal cells.

2. A method according to claim 1, wherein the eukaryotic organism is a multicellular organism, in particular a plant, a fungus, or an animal, such as a mammal, in particular a human.

3. A method according to claim 1 or 2, wherein the presence of Cdc6 protein is detected in oocytes prior to germinal vesicle breakdown.

4. A method according to claim 1 or 2, wherein the absence of Cdc6 protein is detected in oocytes having undergone germinal vesicle breakdown, or in polar globules derived from oocytes having undergone germinal vesicle breakdown, or in ovules.

5. A method according to the method of claim 3, comprising a step of detection of the Cdc6 protein with an anti-Cdc6 protein polyclonal or monoclonal antibody.

6. A method according to the method of claim 4, comprising a step of detection of the Cdc6 protein with an anti-Cdc6 protein polyclonal or monoclonal antibody.

7. A method according to claim 6, comprising the following steps:
- sampling a polar globule from an egg, fertilized or not;
- contacting the sampled polar globule or extracts thereof with an anti-Cdc6 polyclonal or monoclonal antibody,
- detecting the presence or the absence of complexes between the Cdc6 protein and the anti-Cdc6 polyclonal or monoclonal antibody.

8. A kit for carrying out the process of claim 7, comprising an anti-Cdc6 polyclonal or monoclonal antibody, optionally a medium in which the sampled polar

globule is extracted for protein preparation, a medium in which the sampled polar globule, or the protein preparation extracted from said polar globule, is contacted with said antibody, a reagent to detect the complex formed between Cdc6 and said antibody, and a control medium containing the Cdc6 protein.

9. A pharmaceutical composition comprising as active substance a protein comprising or consisting of the Cdc6 protein or a part thereof, or a nucleic acid comprising or consisting of a nucleic acid encoding the Cdc6 protein or a part thereof, in association with a pharmaceutically acceptable carrier.

10. A pharmaceutical composition according to claim 9, wherein the nucleic acid is a RNA, in particular a polyadenylated RNA.

11. A pharmaceutical composition according to claim 9 or 10, wherein the Cdc6 protein comprises a sequence corresponding to the human Cdc6 protein sequence, said sequence corresponding in particular to SEQ ID NO: 2, or wherein the nucleic acid encoding the Cdc6 protein comprises a sequence corresponding to the human Cdc6 nucleic acid sequence, said sequence corresponding in particular to SEQ ID NO: 1.

12. The use of a protein comprising or consisting of the Cdc6 protein or a part thereof, or of a nucleic acid comprising or consisting of a nucleic acid encoding the Cdc6 protein or a part thereof, for the manufacture of a medicament for the treatment of infertilities.

13. The use according to claim 12, wherein the nucleic acid is a RNA, in particular a polyadenylated RNA.

14. The use according to claim 12 or 13, wherein the Cdc6 protein comprises a sequence corresponding to the human Cdc6 protein sequence, said sequence corresponding in particular to SEQ ID NO: 2, or wherein the nucleic acid encoding the Cdc6 protein comprises a sequence corresponding to the human Cdc6 nucleic acid sequence, said sequence corresponding in particular to SEQ ID NO: 1.

15. The use of a compound inhibiting Cdc6 protein activity, such as a pharmacological compound, an antisense polynucleotide directed against the mRNA encoding the Cdc6 protein, a siRNA inhibiting Cdc6 protein expression, an anti-Cdc6 protein antibody or fragments thereof, such as a Fab fragment or a scFv fragment, for the manufacture of a contraceptive.

16. The use according to claim 15, wherein the Cdc6 protein corresponds to a human Cdc6 protein.

17. A phytosanitary composition comprising as active substance a protein comprising or consisting of the Cdc6 protein or a part thereof, or a nucleic acid comprising or consisting of a nucleic acid encoding the Cdc6 protein or a part thereof, or a compound inhibiting Cdc6 activity, such as an antisense nucleotide directed against the mRNA encoding the Cdc6 protein, a siRNA inhibiting Cdc6 protein expression, an anti-Cdc6 protein antibody or fragments thereof, such as a Fab fragment or a scFv fragment.

18. A phytosanitary composition according to claim 13, wherein the Cdc6 protein corresponds to a plant Cdc6 protein.

19. The use of a protein comprising or consisting of the Cdc6 protein or a part thereof, or of a nucleic acid comprising or consisting of a nucleic acid encoding the Cdc6 protein or a part thereof, or of a compound inhibiting Cdc6 protein activity, such as an antisense polynucleotide directed against the mRNA encoding the Cdc6 protein, a siRNA inhibiting Cdc6 protein expression, an anti-Cdc6 protein antibody or fragments thereof, such as a Fab fragment or a scFv fragment, for the treatment of a plant or parts thereof, or for the inhibition of plant reproduction.

20. The use according to claim 19, of a protein comprising or consisting of the Cdc6 protein or a part thereof, or a nucleic acid comprising or consisting of a nucleic acid encoding the Cdc6 protein or a part thereof, for the treatment of plant infertility.

21. The use according to claim 19, of a compound inhibiting Cdc6 protein activity, such as an antisense polynucleotide directed against the mRNA encoding the Cdc6 protein, a siRNA inhibiting Cdc6 protein expression, an anti-Cdc6 protein antibody or fragments thereof, such as a Fab fragment or a scFv fragment, as an herbicide.

22. The use according to any of claims 19 to 21, wherein the Cdc6 protein corresponds to a plant Cdc6 protein.

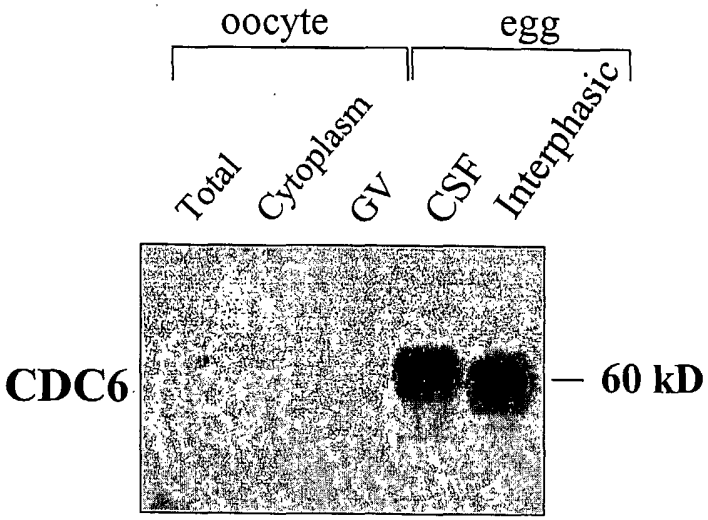
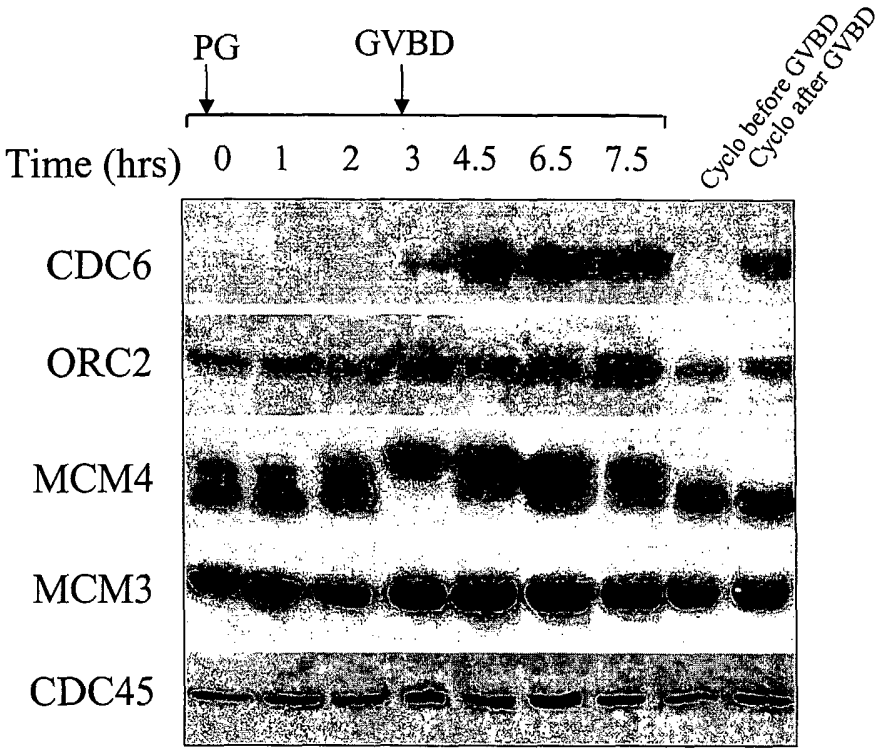


Figure 1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GVBD
Cyclo before GVBD
Cyclo after GVBD
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ORC2
MCM4
MCM3
CDC45

Figure 2

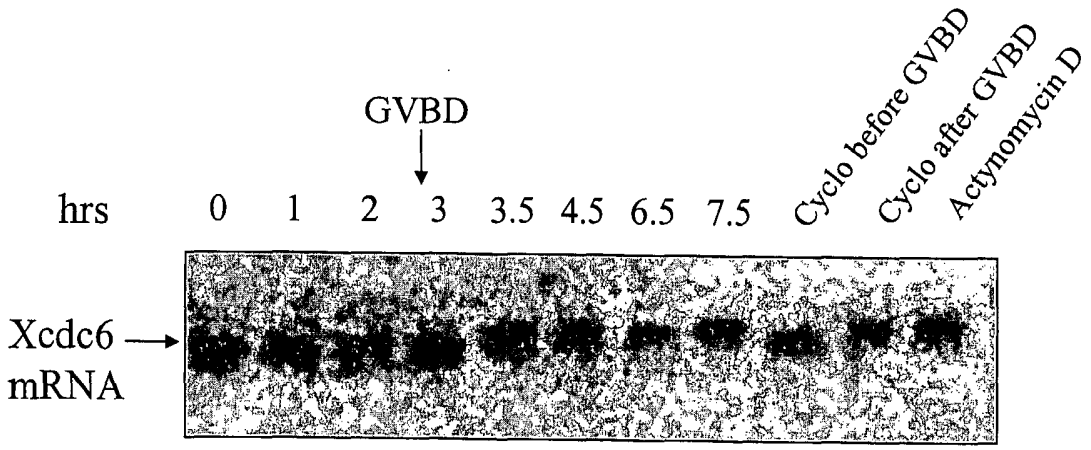
GVBD
hrs
0
1
2
3
3.5
4.5
6.5
7.5
Cyclo before GVBD
Cyclo after GVBD
Actynomycin D
Xcdc6
mRNA

Figure 3

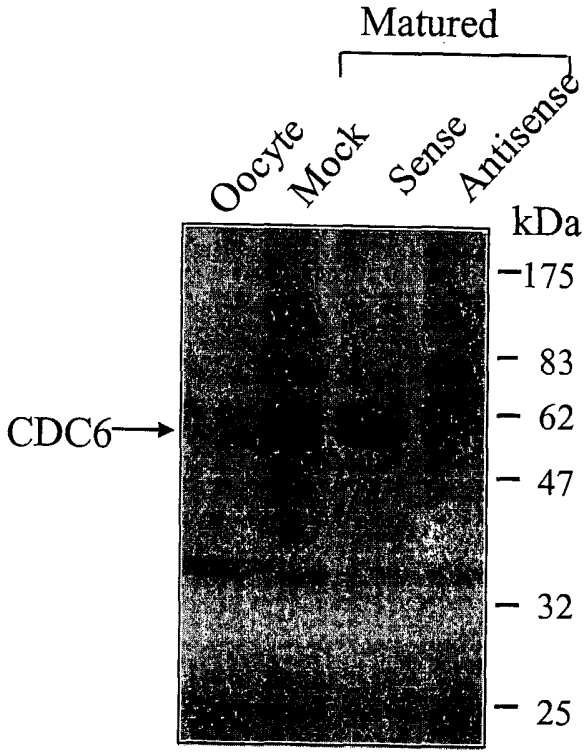
Matured
Oocyte
Mock
Sense
Antisense
kDa
175
83
62
47
32
25
CDC6

Figure 4

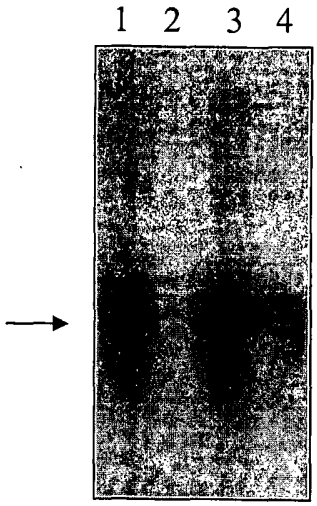

Figure 5A

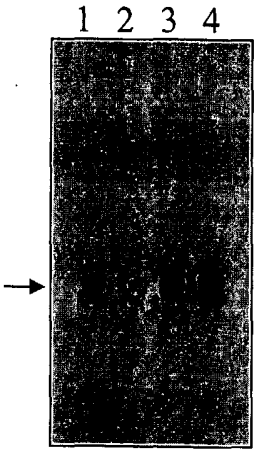

Figure 5B

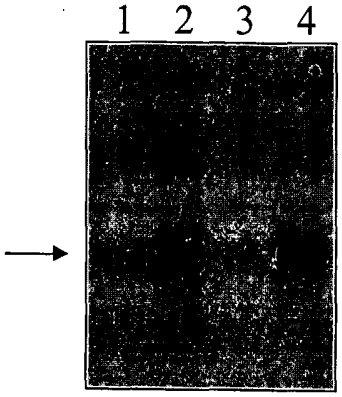

Figure 5C

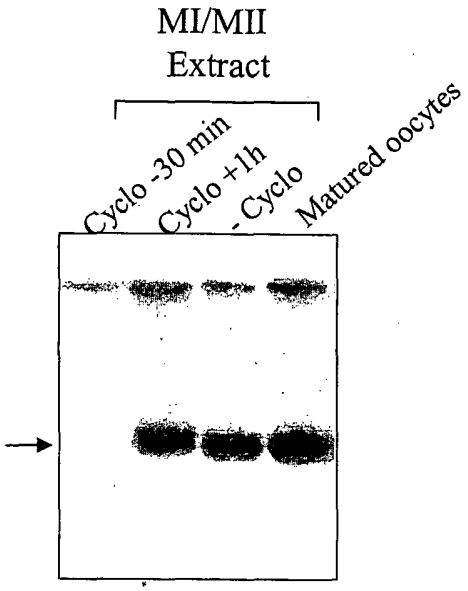

Figure 6A

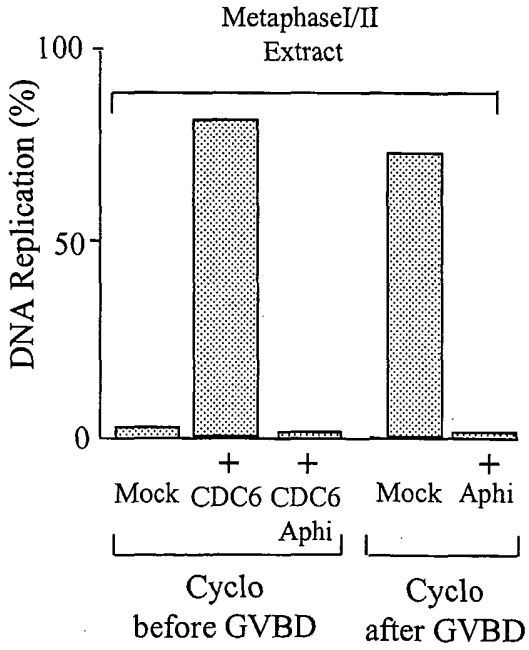

Figure 6B

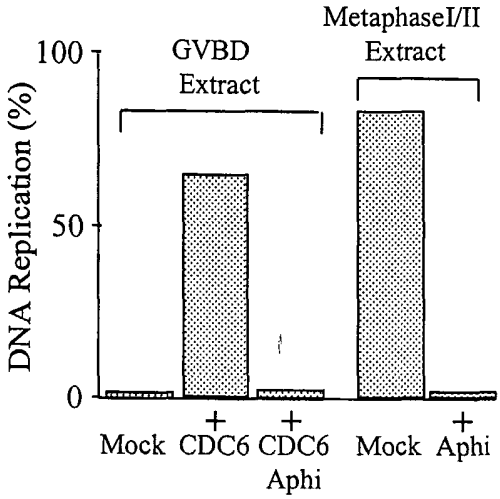

Figure 6C

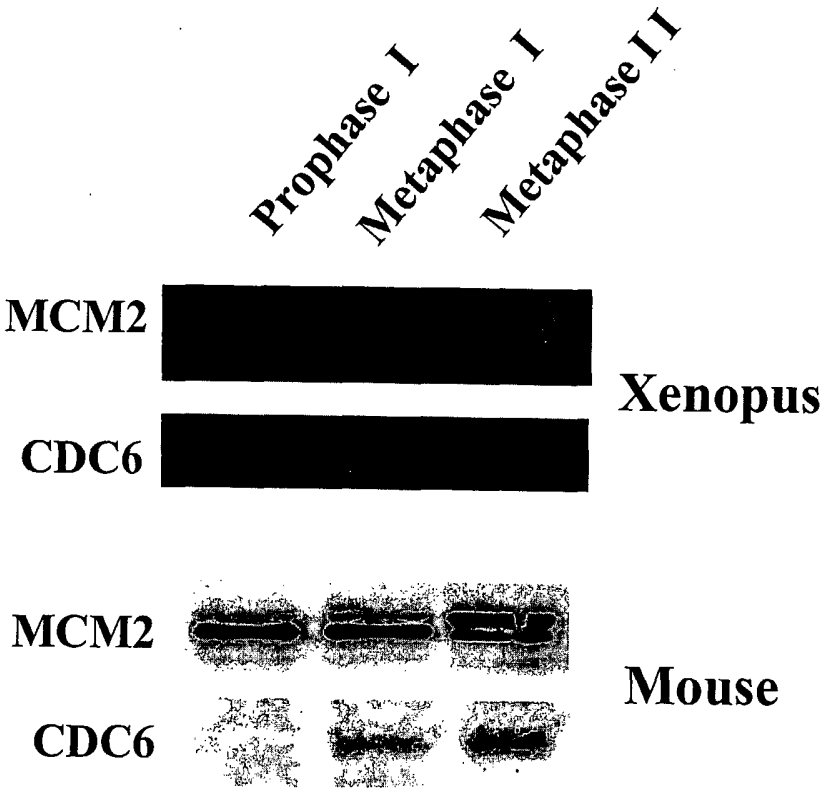
Prophase I
Metaphase I
Metaphase II
MCM2
CDC6
Xenopus
MCM2
CDC6
Mouse

Figure 7

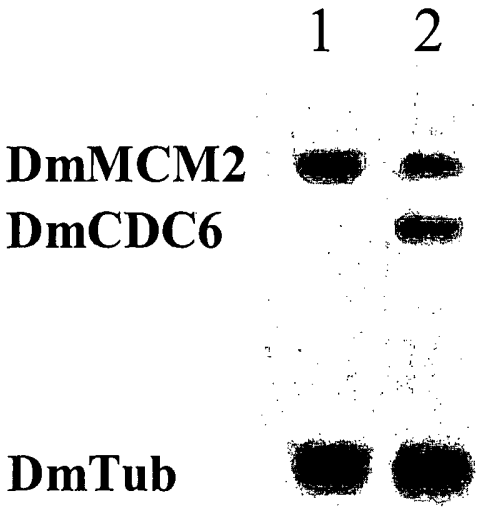

Figure 8

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        35                  40                  45

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Trp Leu Ser Asn Ser His Leu Val Leu Ile Gly Ile Ala Asn Thr Leu
305                 310                 315                 320

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465                 470                 475                 480

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Lys Leu Glu Pro Thr Asn Val Gln Thr Val Thr Cys Ser Pro Arg Val
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65                  70                  75                  80

Lys Glu Asn Gly Pro Pro His Ser His Thr Leu Lys Gly Arg Arg Leu
                85                  90                  95

Val Phe Asp Asn Gln Leu Thr Ile Lys Ser Pro Ser Lys Arg Glu Leu
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           100                 105                 110

Ala Lys Val His Gln Asn Lys Ile Leu Ser Ser Val Arg Lys Ser Gln
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Glu Ile Thr Thr Asn Ser Glu Gln Arg Cys Pro Leu Lys Lys Glu Ser
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Ala Cys Val Arg Leu Phe Lys Gln Glu Gly Thr Cys Tyr Gln Gln Ala
145                 150                 155                 160

Lys Leu Val Leu Asn Thr Ala Val Pro Asp Arg Leu Pro Ala Arg Glu
                165                 170                 175

Arg Glu Met Asp Val Ile Arg Asn Phe Leu Arg Glu His Ile Cys Gly
            180                 185                 190

Lys Lys Ala Gly Ser Leu Tyr Leu Ser Gly Ala Pro Gly Thr Gly Lys
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Thr Ala Cys Leu Ser Arg Ile Leu Gln Asp Leu Lys Lys Glu Leu Lys
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Gly Phe Lys Thr Ile Met Leu Asn Cys Met Ser Leu Arg Thr Ala Gln
225                 230                 235                 240

Ala Val Phe Pro Ala Ile Ala Gln Glu Ile Cys Gln Glu Glu Val Ser
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Arg Pro Ala Gly Lys Asp Met Met Arg Lys Leu Glu Lys His Met Thr
            260                 265                 270

Ala Glu Lys Gly Pro Met Ile Val Leu Val Leu Asp Glu Met Asp Gln
        275                 280                 285

Leu Asp Ser Lys Gly Gln Asp Val Leu Tyr Thr Leu Phe Glu Trp Pro
    290                 295                 300

Trp Leu Ser Asn Ser His Leu Val Leu Ile Gly Ile Ala Asn Thr Leu
305                 310                 315                 320

Asp Leu Thr Asp Arg Ile Leu Pro Arg Leu Gln Ala Arg Glu Lys Cys
                325                 330                 335

Lys Pro Gln Leu Leu Asn Phe Pro Pro Tyr Thr Arg Asn Gln Ile Val
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Thr Ile Leu Gln Asp Arg Leu Asn Gln Val Ser Arg Asp Gln Val Leu
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Asp Asn Ala Ala Val Gln Phe Cys Ala Arg Lys Val Ser Ala Val Ser
370 375 380

Gly Asp Val Arg Lys Ala Leu Asp Val Cys Arg Arg Ala Ile Glu Ile
385 390 395 400

Val Glu Ser Asp Val Lys Ser Gln Thr Ile Leu Lys Pro Leu Ser Glu
405 410 415

Cys Lys Ser Pro Ser Glu Pro Leu Ile Pro Lys Arg Val Gly Leu Ile
420 425 430

His Ile Ser Gln Val Ile Ser Glu Val Asp Gly Asn Arg Met Thr Leu
435 440 445

Ser Gln Glu Gly Ala Gln Asp Ser Phe Pro Leu Gln Gln Lys Ile Leu
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Val Cys Ser Leu Met Leu Leu Ile Arg Gln Leu Lys Ile Lys Glu Val
465 470 475 480

Thr Leu Gly Lys Leu Tyr Glu Ala Tyr Ser Lys Val Cys Arg Lys Gln
485 490 495

Gln Val Ala Ala Val Asp Gln Ser Glu Cys Leu Ser Leu Ser Gly Leu
500 505 510

Leu Glu Ala Arg Gly Ile Leu Gly Leu Lys Arg Asn Lys Glu Thr Arg
515 520 525

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Leu Lys Asp Lys Ala Leu Ile Gly Asn Ile Leu Ala Thr Gly Leu Pro
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<223> Sense oligonucleotide

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<210> 4
<211> 25
<212> DNA
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<220>
<223> Antisense oligonucleotide

<400> 4
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INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 03/07631

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 G01N33/68 C12N9/12 C12N15/82

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 G01N C12N A01H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
BIOSIS, EMBASE, MEDLINE, EPO-Internal, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	TIAN JINGDONG ET AL: "Xenopus Cdc6 confers sperm binding competence to oocytes without inducing their maturation" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES, vol. 94, no. 20, 1997, pages 10729-10734, XP002259845 1997 ISSN: 0027-8424 abstract --- WO 00 26242 A (MENDEZ JUAN R ;STILLMAN BRUCE (US); COLD SPRING HARBOR LAB (US); U) 11 May 2000 (2000-05-11) claims 18-38 --- -/--	1-7,9-17 8-11,17

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
30 October 2003	17/11/2003

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Stricker, J-E

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
P,X	WHITMIRE ELIZABETH ET AL: "Cdc6 synthesis regulates replication competence in Xenopus oocytes" NATURE (LONDON), vol. 419, no. 6908, 17 October 2002 (2002-10-17), pages 722-725, XP002259846 ISSN: 0028-0836 abstract p. 725, Chap. "Antibodies" ---	1-17
P,X	LEMAITRE JEAN-MARC ET AL: "Competence to replicate in the unfertilized egg is conferred by Cdc6 during meiotic maturation" NATURE (LONDON), vol. 419, no. 6908, 17 October 2002 (2002-10-17), pages 718-722, XP002259847 ISSN: 0028-0836 abstract page 722, column 1, paragraph 2 ---	1-17
P,X	PELIZON CRISTINA: "Down to the origin: Cdc6 protein and the competence to replicate." TRENDS IN CELL BIOLOGY. ENGLAND MAR 2003, vol. 13, no. 3, March 2003 (2003-03), pages 110-113, XP002259848 ISSN: 0962-8924 abstract ---	1-7,9-17
P,A	US 2002/192696 A1 (JONG AMBROSE Y) 19 December 2002 (2002-12-19) claims -----	

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/EP 03/07631

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 0026242 A	11-05-2000	US 6361954 B1	26-03-2002
		AU 1457300 A	22-05-2000
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US 2002192696 A1	19-12-2002	NONE	