Silencing CENPF in bovine preimplantation embryo induces arrest at 8-cell stage

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Abstract

Identification of genes that are important for normal preimplantation development is essential for understanding the basics of early mammalian embryogenesis. In our previous study, we have shown that CENPF (mitosin) is differentially expressed during preimplantation development of bovine embryos. CENPF is a centromere–kinetochore complex protein that plays a crucial role in the cell division of somatic cells. To our best knowledge, no study has yet been done on either bovine model, or oocytes and preimplantation embryos. In this study, we focused on the fate of bovine embryos after injection of CENPF dsRNA into the zygotes. An average decrease of CENPF mRNA abundance by 94.9% or more and an extensive decline in immunofluorescence staining intensity was detected relative to controls. There was no disparity between individual groups in the developmental competence before the 8-cell stage. However, the developmental competence rapidly decreased then and only 28.1% of CENPF dsRNA injected 8-cell embryos were able to develop further (uninjected control: 71.8%; green fluorescent protein dsRNA injected control: 72.0%). In conclusion, these results show that depletion of CENPF mRNA in preimplantation bovine embryos leads to dramatic decrease of developmental competence after embryonic genome activation.


Introduction

Identification of genes that are important for normal preimplantation development of mammals is essential for studying early mammalian embryogenesis. A large number of genes expressed from the embryonic genome during embryonic genome activation (EGA) were identified using all sorts of molecular genetic methods including microarray analysis (Hamatani et al. 2004, Wang et al. 2004, Misirlioglu et al. 2006, Kanka et al. 2009, Vigneault et al. 2009). However, the functions of many transcripts during mammalian embryogenesis are still not known. This study concerns one of these genes encoding CENPF (centromeric protein F; mitosin).

CENPF is a large human protein (>350 kDa; 3113 amino acids), which plays a crucial role in cell division by controlling microtubule dynamics, maintaining chromosome condensation, transcription regulation, and cell cycle progression (Liao et al. 1995, Zhu et al. 1995, Holt et al. 2005, Zhou et al. 2005). It is expressed and localized in a cell-cycle-dependent manner (Liao et al. 1995, Zhu et al. 1995). The protein starts to be expressed in the G1/S phase when it is dispersed in nucleoplasm with the exception of nucleolus (Zhu et al. 1995). During late G2 phase, it also relocates to the inner site of nuclear membrane and consequently to the outer plate of the forming prekinetochore (Liao et al. 1995, Zhu et al. 1995). CENPF is one of the earliest proteins associated with kinetochores (Bomont et al. 2005, Yang et al. 2005, Pouwels et al. 2007) and helps to form the correct kinetochore–microtubule interactions (Yang et al. 2005). The protein remains associated with kinetochores until chromosome segregation when it subsequently relocalizes to the spindle mid-zone and intracellular bridge (Liao et al. 1995, Zhu et al. 1995). CENPF reaches its maximum level at the G2/M transition and is rapidly degraded after the cell division (Zhu et al. 1995). Several studies have shown that the depletion of CENPF in somatic cells prevents correct chromosome alignment, destabilizes the microtubule–kinetochore interaction and weakens the tension between sister centromeres (Bomont et al. 2005, Holt et al. 2005, Yang et al. 2005).

The cell cycle during mammalian preimplantation development is very specific in many ways. The cycle is markedly shortened – especially the G1 phase – and at the same time, mitosis occupies longer part of the cycle (Bolton et al. 1984, Iwamori et al. 2002). The transcription of embryonic genome starts in bovines in the late 8-cell stage (8c; Camous et al. 1986, King et al. 1988, Kopecky et al. 1989, Pavlok et al. 1993) and this event is called EGA. Until EGA, all the mRNAs...
and proteins are of maternal origin (Bilodeau-Goeseels & Schultz 1997). Some authors suggest that there is also a so-called minor genome activation between 1- and 4c, which is followed by major genome activation in the 8c (Memili & First 2000, Jakobsen et al. 2006). We have recently proved that in bovine embryos the transcription of embryonic CENPF starts during major genome activation at late 8c. Until then, all the CENPF mRNA is of maternal origin and its amount gradually decreases from 2- to early 8c. After EGA, the expression level increases again and remains almost the same up until the blastocyst stage (Kanka et al. 2009).

Even though the embryo develops without any need of exogenous mitogens, it is very sensitive to changes of external environment. This is the cause of decreased developmental competence of in vitro produced embryos compared to embryos produced in vivo and a significant negative impact on the offspring (Hales & Barker 2001, DeBaun et al. 2003, Ecker et al. 2004).

In this study, we used the CENPF-specific double-stranded RNA (dsRNA) to silence the corresponding mRNA, so that we could monitor the developmental competence of the embryos and consequently compare the role of CENPF in mammalian preimplantation development with somatic cells.

Results

Effect of CENPF dsRNA injection on embryonic CENPF mRNA expression

To confirm that CENPF expression is needful for correct preimplantation development, we employed the RNA interference (RNAi) method. The dsRNA used was homologous to nucleotides 8971–9383 at the 3’ end of bovine CENPF mRNA. The microinjection of CENPF dsRNA efficiently and specifically causes degradation of CENPF mRNA in bovine preimplantation embryos (Fig. 1).

At late 8c, the CENPF mRNA was reduced by 96.0% (P<0.001) in comparison to un.injected control and by 94.9% (P<0.001) in comparison to green fluorescent protein (GFP) dsRNA injected control (Fig. 1A). At late 16-cell stage (16c), the CENPF mRNA was reduced by 97.8% (P<0.02) in comparison to uninjected control and by 98.5% (P<0.002) in comparison to GFP dsRNA injected control (Fig. 1B). No significant difference was found in the abundance of CENPF mRNA between the un.injected group and the GFP dsRNA injected group (P>0.05).

To verify the specificity of CENPF mRNA degradation, we measured the level of mRNA of two control genes: H2A histone family, member Z (H2AFZ) and nucleophosmin (NPM1). No significant distinction between individual groups was detected (P>0.05 in each case; Fig. 2).

Effect of CENPF dsRNA injection on protein expression

To monitor the effect of CENPF mRNA silencing on protein expression, we performed the immunofluorescence analysis using the polyclonal anti-CENPF antibody specific for C-terminus of the protein (Fig. 3). In un injected embryos and embryos injected with GFP dsRNA, CENPF clearly colocalizes with the nuclei of blastomeres (Fig. 3A and B). In CENPF dsRNA injected embryos, we did not detect a similar localization pattern and fluorescence intensity was dramatically decreased (Fig. 3C).

Effect of CENPF mRNA silencing on developmental competence of the embryo

We monitored the number of embryos arrested at individual developmental stages in each treatment group. No developmental impairment was noticed until EGA (8c; P>0.05 in each case; Fig. 4). However, a significantly lower number of CENPF dsRNA injected 8-cell embryos (8c) was capable to develop to 16c or beyond when compared with control groups (mean ± S.E.M.: uninjected control: 71.8%±3.55; GFP dsRNA injected control: 72.0%±2.62; CENPF dsRNA injected group: 28.1%±6.19; P<0.001 in both cases; Fig. 5). Moreover, the embryos in both control groups were of

![Figure 1](https://via.placeholder.com/150)

**Figure 1** Relative abundance of CENPF mRNA after injection of CENPF dsRNA. CENPF mRNA expression (A) in 8-cell stage embryos and (B) in 16-cell stage embryos. The relative abundance (y-axis) represents the amount of CENPF mRNA in a single embryo normalized to one blastomere. Bars show mean ± S.D. a,bValues with different superscripts indicate statistical significance (P<0.05).

![Figure 2](https://via.placeholder.com/150)

**Figure 2** The expression of control genes after injection of CENPF dsRNA. The relative abundance of (A) NPM1 mRNA and (B) H2AFZ mRNA. The relative abundance (y-axis) represents the amount of mRNA in a single embryo normalized to one blastomere. Bars show mean ± S.D.

![Figure 3](https://via.placeholder.com/150)

**Figure 3** Immunofluorescence analysis using the polyclonal anti-CENPF antibody specific (A) for C-terminal, and (B) the antibody specific for N-terminal of the protein. Two control samples: (A) GFP dsRNA and (B) uninjected control. (C) CENPF dsRNA injected group. The fluorescence intensity was dramatically decreased.

![Figure 4](https://via.placeholder.com/150)

**Figure 4** Developmental competence of the embryos after injection of CENPF dsRNA. Bars show mean ± S.D. Significant differences (P<0.05) were found in comparison to uninjected control.

![Figure 5](https://via.placeholder.com/150)

**Figure 5** Developmental competence of the embryos after injection of CENPF mRNA. Bars show mean ± S.D. Significant differences (P<0.05) were found in comparison to uninjected control.
higher morphological quality. The most frequent defect in CENPF dsRNA injected embryos was an unequal size of blastomeres, indistinct boundaries of blastomeres and partial transparency of blastomeres. Immunofluorescence analysis revealed that some of the CENPF dsRNA injected embryos had fragmented nuclei or even the blastomeres did not have any nucleus (Fig. 3C). Only 33.02% ± 3.684 (mean ± S.E.M.) of CENPF dsRNA injected embryos corresponded to the appropriate phenotype, whilst 68.88% ± 6.26 of uninjected embryos and 69.38% ± 10.38 GFP dsRNA injected embryos were of high quality (P < 0.05 in both cases).

**CENPF is not degraded before EGA in bovine preimplantation embryos**

To find out whether CENPF is cyclically degraded and resynthesized in pre-EGA embryos, we blocked the protein synthesis using translation inhibitor cycloheximide (CHX) during cultivation from late 4c to 8c embryos (4c–8c group) and from late 8c to 16c (8c–16c group). The embryos were then examined for CENPF presence using immunofluorescence analysis. No significant difference in staining intensity was found between CHX-treated and non-treated embryos in 4c–8c group (Fig. 6A and B). This suggests that CENPF is not degraded at the end of cell cycle in bovine preimplantation embryos before EGA. However, the results were considerably different in 8c–16c group. We did not observe complete degradation of CENPF, though, but the staining intensity was markedly weaker and the protein was not present in all the nuclei (Fig. 6C and D).

**The localization of CENPF in bovine preimplantation embryos after EGA is cell cycle dependent**

We employed immunofluorescence analysis for the monitoring of CENPF localization during early embryo cell cycle. Our data suggest that bovine CENPF is expressed and localized in the same pattern as the human protein. Most of the blastomeres of the analysed embryos were in interphase. In consistence with the immunofluorescence staining in somatic cells (Liao et al. 1995, Zhu et al. 1995, Hussein & Taylor 2002, Feng et al. 2006), CENPF was detected in the whole nucleus except nucleoli (Fig. 7A and C; non-marked blastomeres). As the chromosomes gradually condense, fluorescent dots begin to appear on chromosomes (Fig. 7A; blastomere marked by arrowhead and B), which is consistent with the kinetochore localization during prophase and prometaphase in somatic cells.
because of the similarity of human and bovine preimplantation development (Tellierd et al. 1990, Adjaye et al. 2007, Kues et al. 2008).

The RNAi using dsRNA was used to silence the CENPF expression. The RNAi causes specific degradation of mRNA and is in fact the only applicable method for studying gene function in early mammalian embryo. Since the quality of early mammalian embryo unwinds from the quality of oocyte and the internal maternal environment, it is not feasible to use knockout for our purpose. For the assessment of degradation efficiency in single embryos we employed the FastLane Cell SYBR Green Kit (Qiagen) and the procedure that was first used by P Šolc for analysing single oocytes (personal communication; details described in Materials and Methods).

The absolute majority of studies concerning CENPF have been done on human somatic cells. Only four of its orthologs – murine CENPF (Goodwin et al. 1999, Ashe et al. 2003, Dees et al. 2005, Soukolis et al. 2005, Evans et al. 2007), avian CMF1 (Wei et al. 1996, Redkar et al. 2002) and worm hcp1 and hcp2 (Cheeseeman et al. 2005, Hajeri et al. 2008) have been studied slightly more intensively. However, these proteins are expressed throughout the whole cell cycle, do not strictly localize to the nucleus or have a somewhat different function (Wei et al. 1996, Goodwin et al. 1999, Redkar et al. 2002, Cheeseeman et al. 2005, Dees et al. 2005, Soukolis et al. 2005, Evans et al. 2007, Hajeri et al. 2008). This suggests that bovine CENPF does not necessarily have to be expressed in a cell-cycle-dependent manner. Moreover, some of the physiological activators and inhibitors of somatic cell cycle do not play the same role during preimplantation development and may even not be needed (Iwamori et al. 2002).

In somatic cells, CENPF participates in the kinetochore–microtubule interaction and is required for chromosome condensation, alignment and segregation. The silencing of CENPF in human somatic cells causes weakened centromere cohesion, premature chromosome decondensation and aneuploidy or metaphase arrest (Bomont et al. 2005, Holt et al. 2005, Yang et al. 2005).

The immunofluorescence analysis of bovine preimplantation embryo at the 16c exhibits the same expression and localization of CENPF as human somatic cells (Liao et al. 1995, Zhu et al. 1995, Hussein & Taylor 2002, Feng et al. 2006). In non-mitotic blastomeres we detected CENPF dispersed in nucleoplasm; in prophase and prometaphase blastomeres distinct foci of CENPF staining were detected on chromosomes. The protein was not detectable at the end of mitosis in preimplantation embryos after EGA (Fig. 7C). In somatic cells CENPF is degraded at the end of mitosis (Zhu et al. 1995). However, in preimplantation embryos before EGA, we did not detect any observable fall of protein amount after 24 h treatment with translation inhibitor CHX, which
poses a sufficiently long period for passing through the cell cycle (Fig. 6A and B). On the other hand, after CHX treatment of embryos post EGA, we detected a considerable decline of staining intensity (Fig. 6C and D). Hence, we suppose the preservation of maternal CENPF protein until the EGA in the late 8c. On the basis of the data mentioned above, we supposed that the CENPF-depleted embryos might arrest after EGA.

To confirm this, we performed the dsRNA mediated specific degradation of CENPF mRNA. We did not notice any differences in developmental competence between individual treatment groups until the 8c (P > 0.05 in each case; Fig. 4). However, the developmental competence of CENPF dsRNA injected embryos steeply decreased after the 8c; lesser than one-third of 8c embryos reached the 16c (Fig. 5) and only rare embryo-survivors further developed. Similarly, POU5F1 (Oct-4)-depleted embryos are able to develop until the morula stage without any significant differences in developmental competence, although the maternal mRNA can be detected in embryos before EGA and the first embryonic transcript in bovine can be detected at the morula stage (Nganvongpanit et al. 2006a, 2006b). In our previous study (Kanka et al. 2009), we have shown that CENPF transcription from embryonic genome is activated during major genome activation, i.e. at late 8c. The arrest of CENPF-mRNA-depleted embryos just at the 8c suggests that until then, the embryos utilize maternal reserves of the protein. In addition, this is in agreement with our CHX experimental data.

In the CENPF dsRNA injected 16c embryos, the CENPF mRNA was silenced too. This suggests that embryos are to some extent able to compensate the decreased level of CENPF mRNA. The depletion of CENPF in somatic cells causes incorrect distribution of chromosomes during mitosis, since the cells form an interaction that is too weak between kinetochores and microtubules (Holt et al. 2005, Yang et al. 2005, Feng et al. 2006). Some of the CENPF-depleted cells progress through mitosis without chromosome segregation in anaphase or form a tripolar spindle resulting in multinucleated cells or aneuploidies (Holt et al. 2005, Yang et al. 2005, Feng et al. 2006).
Feng et al. 2006). Most of the CENPF-depleted cells do not continue the cell-cycle progress (Holt et al. 2005, Yang et al. 2005) and undergo apoptosis (Yang et al. 2005). However, a minority of cells are able to progress through the cell cycle despite the premature mitotic exit with unaligned chromosomes (Holt et al. 2005). The progress of some of the cells is probably caused by insufficient function of the mitotic checkpoint, which is able to delay, but not arrest, the progress (Feng et al. 2006). From our immunofluorescence data it follows that the nuclei of CENPF dsRNA injected embryos are in many cases fragmented, or the number of nuclei is less than the number of blastomeres (Fig. 3C). This suggests that also in blastomeres the cell cycle progress is arrested. However, some of the blastomeres are able to develop further. Moreover, the results of different studies are not homogeneous as to the cell fate after CENPF silencing (Holt et al. 2005, Laoukili et al. 2005, Yang et al. 2005, Feng et al. 2006). Ma et al. (2006) suggest that this may be caused by different efficiencies of the mRNA silencing. We, however, agree with the hypothesis of Feng et al. (2006), who assumed that the variation in results might be caused by the usage of different cell lines. Since the embryonic cells are strongly forced to cell division, they are supposed to surmount the cell-cycle-arrest signals quite easily.

In conclusion, we showed that the introduction of CENPF-specific dsRNA into the zygote leads to mRNA and protein silencing in preimplantation development. The inhibition of CENPF mRNA results in considerable deterioration in developmental competence after achieving the 8c and arrest of the majority of embryos before reaching the 16c. These findings are in agreement with data acquired on human somatic cells and indicate that after activation of embryonic genome transcription, CENPF is expressed and localized in the same way as in human somatic cells and that the expression of CENPF mRNA is necessary for proper course of preimplantation development.

Materials and Methods

**IVF and embryo culture**

Unless otherwise indicated, chemicals were purchased from Sigma (Sigma–Aldrich) and plastic from Nunclon (Nunc, Roskilde, Denmark).

Bovine embryos were obtained after in vitro maturation of oocytes and their subsequent fertilization and culture in vitro. Briefly, abattoir derived ovaries from cows and heifers were collected and transported in thermo containers in sterile saline at about 33 °C. The follicles with diameter between 5 and 10 mm were dissected with fine scissors and then punctured. The cumulus–oocyte complexes were evaluated and selected according to the morphology of cumulus and submitted to in vitro maturation in TCM 199 supplemented with 20 mM sodium pyruvate, 50 U/ml penicillin, 50 μg/ml streptomycin, 10% estrus cow serum (ECS) and gonadotropins (P.G. 600, 15 U/ml; Intervet, Boxmeer, Holland) without oil overlay in 4-well dishes under atmosphere of 5% CO₂–7% O₂–88% N₂ at 39 °C for 24 h.

For IVF, the cumulus–oocyte complexes were washed four times in PBS and once in fertilization medium (TALP) and transferred in groups of up to 40 into 4-well dishes containing 250 μl of TALP per well. The TALP medium contained 1.5 mg/ml BSA, 30 μg/ml heparin, 0.25 mM sodium pyruvate, 10 mM lactate and 20 μM penicillamine. Cumulus–oocyte complexes were then co-incubated with frozen-thawed wash...
semen from one bull previously tested in the IVF system. Viable spermatozoa were washed in TALP and centrifuged at 100 g for 5 min. Spermatozoa were counted in a haemocytometer and diluted in the appropriate volume of TALP to give a concentration of \(2 \times 10^6\) spermatozoa/ml. A 250 µl aliquot of this suspension was added to each fertilization well to obtain a final concentration of \(1 \times 10^6\) spermatozoa/ml. Plates were incubated for 20 h at 39 °C under an atmosphere composed of 5% CO\(_2\)–7% O\(_2\)–88% N\(_2\).

At 20 h post fertilization (hpf) zygotes were denuded by gentle pipetting, and transferred to B2 Menezo medium supplemented with 10% ECS and cultured in an atmosphere of 5% CO\(_2\)–7% O\(_2\)–88% N\(_2\) at maximum humidity (25 zygotes in 25 µl of medium under mineral oil; COOK, Eight Mile Plains, Queensland, Australia). The dishes were examined at 32, 44, 56, 92 and 120 hpf and 2-cell, 4-cell, early 8-cell, late 8-cell embryo and morula were collected at each time point respectively.

**The CHX treatment**

To block the protein synthesis CHX (Sigma–Aldrich) was added to the culture medium at a final concentration of 10 µg/ml 48 and 80 hpf respectively. After 24 h cultivation, the embryos were washed in PBS and fixed for immunofluorescence. In total, 84 embryos were included in the study in four independent experiments – 48 hpf: 27 CHX-treated embryos and 16 controls; 80 hpf: 22 CHX-treated embryos and 19 controls were immunofluorescently examined.

**Synthesis of DNA template**

The DNA template for RT-PCR was isolated from bovine embryonic fibroblasts using RNeasy Mini Kit (Qiagen). The template was synthesized using primers ‘CENPF dsRNA’ (see Table 1). The identity of fibroblastic and embryonic sequence was verified by sequencing. These primers generated amplicons corresponding to the bovine cDNA sequences in GenBank (XM_612376) and were fused with the T7 promoter. The template was synthesized using primers ‘CENPF’ dsRNA (5’ AGGATCCCTATAGCTCCTATAGGGA-GAGGAGGCTTCCGAAAGTTGAAA3’, 5’ ACTCGAGTAATACGACTCACTATAGGGAGTGCTG3’, 5’ CCAGCTGTTGGTTTGGAGG3’). The identity was confirmed by sequencing. The template was transcribed using MEGAscript RNAi Kit (Ambion). An amount of 1 µg of DNA template was used for each reaction. The reaction mixture was incubated for 5 h at 37 °C and the sense and antisense strands were transcribed in the same reaction. To anneal them, the sample was incubated at 75 °C for 5 min and then left to cool at room temperature. The residual DNA template and ssRNA were digested and the dsRNA was purified according to the manufacturer’s instructions. One microlitre of RNA acquired by in vitro transcription and 1 µl of final dsRNA were resolved by electrophoresis on 1.5% agarose gel to confirm the integrity of the dsRNA and efficiency of the annealing step.

**Synthesis of dsRNA**

The DNA template coupled with T7 promoter was in vitro transcribed using MEGAscript RNAi Kit (Ambion). An amount of 1 µg of DNA template was used for each reaction. The reaction mixture was incubated for 5 h at 37 °C and the sense and antisense strands were transcribed in the same reaction. To anneal them, the sample was incubated at 75 °C for 5 min and then left to cool at room temperature. The residual DNA template and ssRNA were digested and the dsRNA was purified according to the manufacturer’s instructions. One microlitre of RNA acquired by in vitro transcription and 1 µl of final dsRNA were resolved by electrophoresis on 1.5% agarose gel to confirm the integrity of the dsRNA and efficiency of the annealing step.

**Zygote microinjection**

Good quality zygotes were injected 20 hpf at the stage of two pronuclei. dsRNAs were dissolved in RNase-free water to a final concentration of 800 ng/µl. Zygotes were microinjected with ~5 pl of the dsRNA using an M-5000 micromanipulator (Burleigh, Exfo Life Sciences, Mississauga, Ontario, Canada) and PM 2000B4 microinjector (MicroData Instrument, South Plainfield, NJ, USA). Pipettes for microinjection were made using P97 Pipette Puller (Sutter Instrument Company, Novato, CA, USA). Two control groups were established – the uninjected group and a group injected with GFP dsRNA. The microinjection medium was Whitten’s medium supplemented with 10 mmol/l HEPES (pH 7.3).

In total, 839 embryos were included in the study in 12 independent injection sessions. Embryos were categorized into the following groups: 1) embryos injected with CENPF dsRNA (266 embryos), 2) embryos injected with GFP dsRNA (237 embryos), and 3) uninjected embryos (336 embryos).

**Table 1** Details of primers used for quantitative RT-PCR and double-stranded RNA synthesis.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequences</th>
<th>Annealing temperature (°C)</th>
<th>Amplicon size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CENPF (XM_612376) dsRNA</td>
<td>5’ AGGATCCCTATAGCTCCTATAGGGA-GAGGAGGCTTCCGAAAGTTGAAA3’, 5’ ACTCGAGTAATACGACTCACTATAGGGAGTGCTG3’, 5’ CCAGCTGTTGGTTTGGAGG3’</td>
<td>50</td>
<td>413</td>
</tr>
<tr>
<td>GFP dsRNA* (Anger et al. 2005)</td>
<td>5’ AGGATCCCTATAGCTCCTATAGGGA-GAGGAGGCTTCCGAAAGTTGAAA3’, 5’ ACTCGAGTAATACGACTCACTATAGGGAGTGCTG3’, 5’ CCAGCTGTTGGTTTGGAGG3’</td>
<td>55</td>
<td>712</td>
</tr>
<tr>
<td>CENPF (XM_612376) mRNA quantification</td>
<td>5’ TTGTAAGAAAGGGTTGGC3’, 5’ CCAAGCTTTGTTGGGAGG3’</td>
<td>50</td>
<td>172</td>
</tr>
<tr>
<td>NPM1 (XM_001252818) mRNA quantification</td>
<td>5’ ACAGCCAAGGTTCTTCCTT3’, 5’ TTCCACCTTCTCCCTCTCTCTCTC3’</td>
<td>55</td>
<td>154</td>
</tr>
<tr>
<td>H2AFZ (NM_174809) mRNA quantification</td>
<td>5’ AGGAGGAGTGCCATGAGGAGT3’, 5’ CCAACACCGAATTGAGCTTG3’</td>
<td>60</td>
<td>208</td>
</tr>
</tbody>
</table>

*Transcribed from empty p-Bluescript-GFP vector; kindly donated by M Anger and P Solc.
After microinjection, embryos were cultivated under the conditions mentioned above and collected at specific developmental stages (late 8c – day 3.5 post fertilization, late 16c – day 4.5 post fertilization). The number of embryos that reach each developmental stage was counted and the morphological state of each embryo was determined using phase-contrast technique.

**Monitoring of mRNA degradation efficiency**

The embryos were washed and lysed using FastLane Cell SYBR Green Kit (Qiagen). Single embryos were washed in DMEM without foetal bovine serum and supplemented with 2% (w/v) polyvinylalcohol (PVA), PBS supplemented with 2% (w/v) PVA and FCW buffer (a component of FastLane Cell SYBR Green Kit) and stored dry and deep-frozen at −80°C until used. Whole single embryos were lysed in 10 µl of the mixture of Buffer FCPL and gDNA Wipeout Buffer 2 (both members of FastLane Cell RT-PCR kit; Qiagen) according to the manufacturer’s instructions and the lysate was directly used for the RT-PCR. Quantitative RT-PCR was performed using One Step RT-PCR kit (Qiagen). The samples were incubated at 50°C for 30 min and heated at 95°C followed by 45 cycles of 94°C 20 s, 50°C 20 s and 72°C 30 s. The final extension step was held for 10 min at 72°C. The RT-PCR data were normalized to the number of blastomeres.

The experiments were carried out on Rotor-Gene 3000 (Corbett Research, Mortlake, New South Wales, Australia). Fluorescence data were acquired at 3°C below the melting temperature to distinguish the possible primer dimers; the standard curve was created by Internal Rotor-Gene software (Corbett Research, Mortlake, Australia). The starting amount of corresponding RNA in analysed samples was determined by appointing the Cts to the curve. Products were verified by melting analysis and gel electrophoresis on 1.5% agarose gel with ethidium bromide staining. The experiment was repeated four times.

**Immunofluorescence**

Embryos were fixed in 4% paraformaldehyde supplemented with 1% (v/v) Triton X-100 for 50 min at 4°C. Fixed embryos were processed immediately or stored in PBS up to 3 weeks at 4°C. After washing in PBS embryos were incubated in 1% (v/v) Triton X-100 for 15 min. All subsequent steps were done in PBS supplemented with BSA (0.25% for mouse anti-CENPF antibody against N-terminus – BD Biosciences, Erembodegem, Belgium; 0.4% for rabbit anti-CENPF antibody against C-terminus – Novus Biologicals, Littleton, CO, USA) and 0.05% (w/v) saponin (PBS/BSA/sap). Embryos were blocked with 2% (v/v) normal goat serum for 1 h and incubated with primary antibody in PBS/BSA/sap overnight at 4°C (mouse anti-CENPF antibody against N-terminus – 1:100; rabbit anti-CENPF antibody against C-terminus – 1:1000). After thorough washing the embryos were incubated with goat anti-mouse antibody conjugated with Alexa 594 (Invitrogen) or goat anti-rabbit antibody conjugated with FITC (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or with Alexa 594 (Invitrogen) in PBS/BSA/sap for 1 h at room temperature in the dark. The nuclei were stained and the embryos were mounted on glass slides using VECTASHIELD HardSet Mounting Medium with DAPI (Vector Laboratories, Peterborough, UK). The samples were examined with a confocal laser-scanning microscope Leica TCS SP (Leica Microsystems AG, Wetzlar, Germany). Controls of immunostaining specificity were carried out by omitting primary antibody or using another species-specific secondary antibody conjugate. The images were processed using the ImageJ software (NIH, Bethesda, MD, USA; http://rsb.info.nih.gov/ij).

**Statistical analyses**

The data were analysed using SigmaStat 3.0 software (Jandel Scientific, San Rafael, CA, USA), the Student’s t-test or Mann–Whitney Rank Sum tests were used. P<0.05 was considered as significant.

**Declaration of interest**

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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