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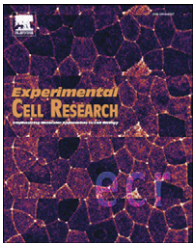

Research Article

Involvement of ZFPIP/Zfp462 in chromatin integrity and survival of P19 pluripotent cells

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ABSTRACT

Toti- or pluripotent cells proliferation and/or differentiation have been shown to be strongly related to nuclear chromatin organization and structure over the last past years.
We have recently identified ZFPIP/Zfp462 as a zinc finger nuclear factor necessary for correct cell division during early embryonic developmental steps of vertebrates. We thus questioned whether this factor was playing a general role during cell division or if it was somehow involved in embryonic cell fate or differentiation.
To achieve this goal, we performed a knock-down experiment in the pluripotent P19 and differentiated 3T3 cell lines, both expressing endogenous ZFPIP/Zfp462. Using specific shRNA directed against *ZFPIP/Zfp462* transcripts, we demonstrated that depletion of this protein induced cell death in P19 but had no effect in 3T3 cells. In addition, in the absence of the protein, the P19 cells exhibited a complete destructuration of pericentromeric domains associated with a redistribution of the HP1α proteins and an increase in DNA satellites transcribed RNAs level.
These data suggested an instrumental role of ZFPIP/Zfp462 in maintaining the chromatin structure of pluripotent cells.

Introduction

We have recently identified *ZFPIP/Zfp462* as a vertebrate-specific gene encoding a nuclear zinc finger protein that strongly interacts with Pbx1 [1]. The 250 kDa ZFPIP/Zfp462 protein contains a strikingly important number (34) of C_2H_2 zinc fingers distributed throughout the sequence and a putative Nuclear Localization Signal (NLS). Previous data have shown that *ZFPIP/Zfp462* mRNA is expressed during development in the mouse with a maximum yield between E10.5 to E12.5. This expression is particularly abundant in brain, somites and limbs buds [1,2]. *ZFPIP/Zfp462* appeared to be an essential developmental gene in *Xenopus laevis*. Indeed, knocked-down expression of *ZFPIP/Zfp462* in *X. laevis* embryos leads to a rapid cell division arrest coupled with important nuclear chromatin disorders: mitotic cells undergo abnormal mitosis, with aberrant metaphase, anaphase, incomplete chromosome segregation or conjoined nuclei [3]. Furthermore, decrease of ZFPIP/Zfp462 levels seems to impair cell cycle, inducing cell death with a mechanism described as "mitotic catastrophe" [4].

Analysis of the chromatin/cell division disturbance in whole animal using morpholinos remains difficult because of the important cellular variability in developing embryos [5]. Therefore, analysis of such mechanism in a cell culture model is an alternative approach. To this aim, we sought for a model of ZFPIP/Zfp462 expressing cell line and selected out the murine embryonal carcinoma P19 cell line which has already been widely used to

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study early events of embryonic differentiation [6,7]. Indeed, these cells resemble the inner cell mass of the early embryo and can differentiate into different cell types according to the culture conditions, such as fibroblastic, muscular or neural cells [8,9].

With this work, we demonstrated that *ZFPIP*/*Zfp462* expression was cell-specific. Indeed, the protein was detected in P19, ES or 3T3 whereas no expression of the gene was detected in several vertebrate cell lines. Using a shRNA approach, we demonstrated that depletion of the protein in the P19 embryonic cells induced abnormal nucleus phenotype that was not observed in differentiated depleted 3T3 cells. Moreover, ZFPIP/Zfp462-depleted P19 cells exhibited disruption of nuclear heterochromatin, correlated to a delocalization of HP1α and an increase of DNA satellites transcription. Following these abnormal nuclear cell processes, the P19 cells died within 24 h.

These results demonstrated that (i) the absence of ZFPIP/Zfp462 led to a disorganization of pericentromeric domains in P19 cells which were subsequently not able to divide correctly and that (ii) the gene appeared vital in P19 pluripotent cells but not in 3T3 cells. The overall data clearly showed that *ZFPIP*/*Zfp462* was an essential gene involved in chromatin structure modelling mechanisms occurring during cell division of pluripotent cells.

Materials and methods

Plasmid constructs

The pCMV-N-ZFPIP and pCMV-C-ZFPIP vectors correspond to the N-terminal and the C-terminal regions (respectively N-ZFPIP and C-ZFPIP) of the human ZFPIP/Zfp462 encoding cDNA cloned into *Bam*HI sites of the pCMVflag6c vector (Sigma) as described in [1]. The full-length human *ZFPIP*/*Zfp462* cDNA was purified from the pT7TS-ZFPIP construct [3] and cloned into *Eco*RV and *Acc*65I sites of the pCMV-flag6b vector (pCMV-FL-ZFPIP).

The eukaryotic expression vector pEGFP-C3 (pGFP) from Clontech was used for expression of the Enhanced Green Fluorescent Protein (EGFP) and then detection of the transfected cells.

The vectors pLKO.1 puroShZFPIP (pSh-ZFPIP) and pLKO.1 puro-ShSCR (pSh-SCR) from Sigma were used for shRNA expression. The pSh-ZFPIP plasmid contains a specific shRNA sequence directed against *ZFPIP*/*Zfp462* mRNA and the pSh-SCR plasmid contains a "Scramble" shRNA which targets neither murine nor human mRNA sequences (Table 1). The DNA vectors contain a puromycin resistance gene as a cell transfection marker. In the system, untransfected cells died rapidly (12–24 h) after addition of puromycin in the culture medium.

RNA extraction and quantitative real-time PCR

Total RNAs were extracted from cells or whole embryos E 9.5 using the Nucleospin kit (Macherey-Nagel). For each RT-PCR reaction, one microgram of total RNA was reverse transcribed to cDNA using random primers hexamers (InVitrogen). Samples along with primers and Syber Green Master Mix (Applied Biosystems) were run in an ABI Prism 7000 SDS (Applied Biosystems) according to the manufacturer's protocol. Relative quantification of *ZFPIP*/*Zfp462* or major-satellite mRNAs in each sample was calculated in comparison of their Ct values previously normalized with Ct values obtained for *HPRT* cDNA amplification. Experiments were run three times from different RNA preparations. The standard deviations reflect variability within triplicates. The primers sequences used in qRT-PCR experiments were indicated in Table 1.

Cell culture

Mouse embryonic carcinoma P19 cell line was a gift from Pr. Gilles Salbert and 3T3 cells were from ATCC. All cells were cultured in Dulbelcco's modified Eagle's medium (DMEM, Gibco, InVitrogen) with 10% fetal calf serum (PAA laboratories) and 5 mg/ml penicillin, 5 mg/ml streptomycin and 10 mg/ml neomycin (InVitrogen). Cells were maintained in a humidified incubator at 37 °C and with 5% CO_2. Pelleted murine ES CK35 cells were a gift from Dr. Michel Cohen-Tanoudji [10].

Protein extracts and western blot analysis

Cells were washed twice with PBS and incubated in SDS-lysis buffer (0.1 M Tris–HCl, pH 7.5; 0.15 M NaCl; 0.1% SDS; 1% Triton X-100; 1% Na deoxycholate) for 30 min on ice. Proteins were separated by electrophoresis using 6-10.5% gradient SDS-Polyacrylamid gel. Proteins were then electro-transferred onto nitrocellulose membrane (Amersham Bioscience). Western blots were performed using anti-β-tubulin and anti-actin monoclonal antibodies (Sigma) or anti-ZFPIP/Zfp462 serum [α-ZFPIP [1]]. The anti-ZFPIP/Zfp462 serum directed against mouse protein also reacts with *Xenopus* and human protein [1,3,11]. Revelations were performed by ECL+ (Amersham Bioscience) according to manufacturer's instructions.

Immunofluorescence and microscopy

For fluorescence microcopy analysis, cells were grown on 18 mm glass coverlips in 12 wells culture plates. Chemical fixation of the cells was performed using 4% paraformaldehyde in PBS for 30 min at room temperature (RT). Cells were washed twice in PBS (2×

Table 1 – qRT-PCR primers and shRNA sequences.

Oligo name	Sense (5′–3′)	Antisense 5′–3′)	Reference
mHPRT	gtcaagggcatatccaacaacaaac	gctggtgaaaaggacctctcg	[1]
mZFPIP	ttctataaaggatgagtttgtgattgc	tcctggactgtggccatagtaac	[1]
Major-satellite	gacgacttgaaaaatgacgaaatc	catattccaggtccttcatgtgc	[39]
Minor-satellite	atcaatgagttacaatgagaaacatggaaa	tgatatacactgttctacaaatcccgtttc	[39]
Sh-SCR	ccggcaacaagatgaagagcaccaactcgagttggtgctcttcatcttgttgttttt	Sigma	
Sh-ZFPIP	ccgggcaggaacgaaatccatacaactcgagttgtatggatttcgttcctgcttttt	Sigma	