Cell Reports

Supplemental Information

Exogenous Expression of Human Protamine 1 (hPrm1) Remodels Fibroblast Nuclei into Spermatid-like Structures

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Supplemental Data

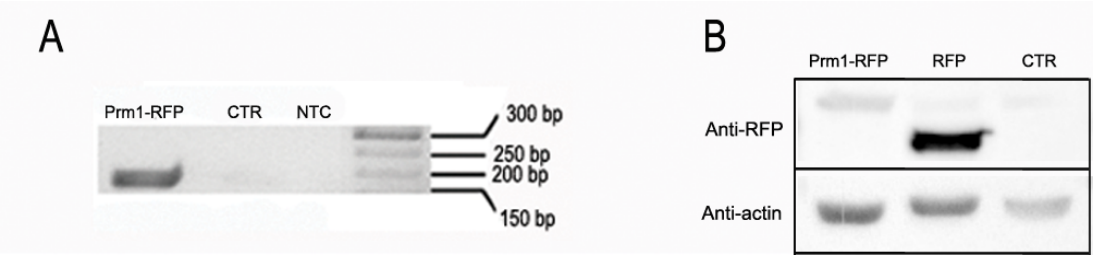

Figure S1. Supplemental Figure 1 related to Figure 1. Confirmation of ectopic Prm1 expression in somatic cells. A - Protamine mRNA expression in sheep adult fibroblasts (RT-PCR). Prm1-RFP: fibroblasts transfected with protamine tagged with RFP. CTR: fibroblasts not transfected. NTC: no template control; B - Protamine protein expression in adult fibroblasts (Western blot). Prm1-RFP: fibroblasts transfected with pPrm1-RFP. pTag-RFP: fibroblasts transfected with empty plasmid-RFP. CTR: fibroblasts not transfected. Immunoblotting with anti-tRFP, anti-actin were used as loading control.

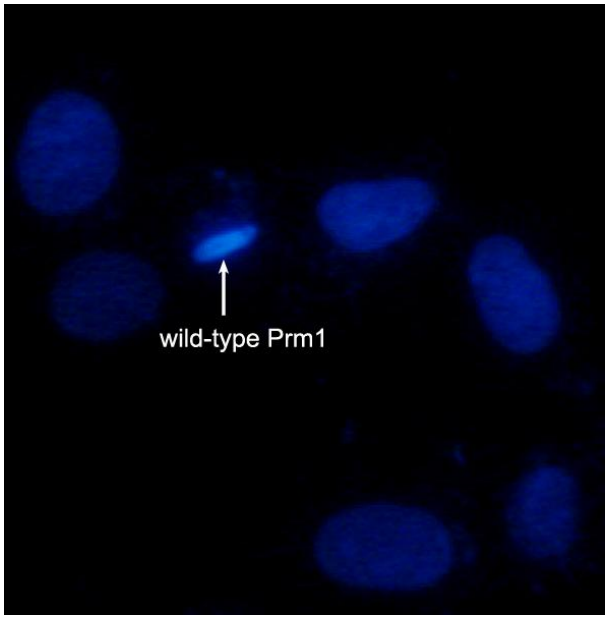

Figure S2. Figure supplemental 2, related to Figure 1. Confirmation that fibroblasts transfected with wild-type Prm1 shows nuclear reorganization similar to the detected in pPrm1-RFP transfected ones. Fibroblasts transfected with wild-type protamine without RFP tag.

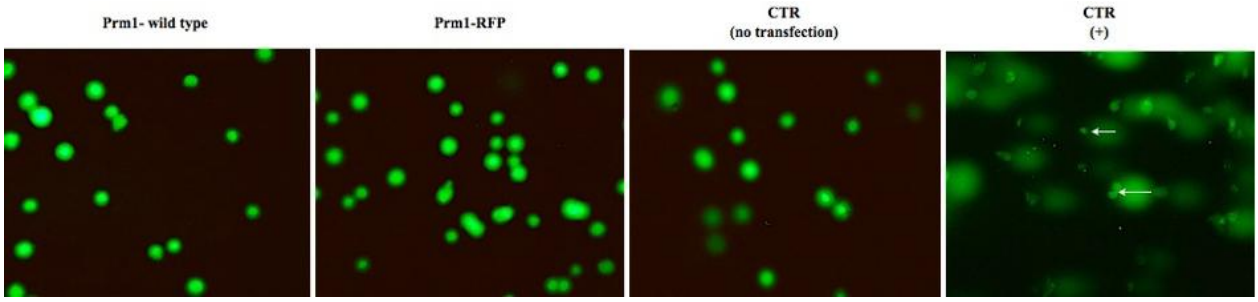

Figure S3. Figure supplemental 3, related to Figure 2J. Comet assay excluded the presence of DNA fragmentation in fully protaminized fibroblasts. Comet assay on Prm1-wild type, Prm1-RFP transfected cells, CTR – un-transfected cells, and CRT (+) fibroblasts treated with H_2O_2.

Table S1. Table supplemental 1, related to Figure 1J. Binding of Prm1 to DNA on 42 DNA binding sites on 10/27 chromosomes (2n=54 in sheep). Binding occurred in 28 gene-rich domains – 14 (50%) genic and 14 (50%) intergenic – while the other 14 were classified as scaffold.

Table S2. **Table supplemental 2, related to Table 1.** Incorporation of Prm1 in somatic nuclei at cell cycle stages.

Cell Cycle	G1	S	G2
CTR	6412/8981 (64.12%)	396/8981 (4.4%)	2173/8981 (24.2%)
PR +	6221/8569 (72.6%)	375/8569 (4.4%)	1973/8569 (23%)

Supplemental Experimental Procedure

All chemicals, unless otherwise indicated, were obtained from Sigma Chemical Co.

RNA isolation and Reverse Transcription

Poly (A) + RNA was isolated from cells using Dynabeads mRNA DIRECT kit (Invitrogen Dynal AS, Oslo, Norway) according to the manufacturer's instructions. RT was performed using 80% of the eluted Poly (A) + RNA in a total volume of 20µl using the QuantiTect Reverse Transcription Kit (Qiagen, Milan, Italy). cDNAs were diluted 1:3 in H_20. The PCR reactions were performed using the PCR Master Mix (Promega, Milano, Italy). PCR conditions: 95° 5' (95°C, 30", 58" 30°C,

72°C 30") x 35 cycles, 72°C 10 '. Primers were: FW; atggccagataccgatgct, RV; cagcatcttcgcctcctc; amplicon: 160bp.

Flow Cytometry for Cell Cycle Assay

Cells transfected with pPrm1 (without TagRFP) were fixed with 70% Ethanol and stained with 5 µg/ml Propidium Iodide. Red fluorescence (>630 nm) (DNA) was measured using a flow cytometer (Beckman coulter, Epics XL). Approximately 100000 cells/test were acquired at a flow rate of 400-500 events/s.

DNA integrity – Comet Assay

DNA integrity was evaluated using the single cell gel electrophoresis assay (CometAssay) (Trevigen, Bologna, Italy) according to the manufacturer's instructions. Briefly, cells were diluted to a concentration of 10^5 cells/ml in PBS. The cells were combined with molten LM agarose at a ratio of: 1:10 (v/v), then 75 µl were placed on comet slides. The slides were put in the dark at 4°C for 10 minutes, then immersed in a pre cooled (4°C) lysis solution for 1 hour at 4°C. Afterward, the slides were immersed in a freshly prepared alkaline solution, which consisted of 0.6g NaOH pellets, 250 µl of 250mM EDTA, pH 10.0 and 49.75ml deionized water, for 60 minutes in the dark at room temperature. Finally, the slides were washed in a TBE buffer (Tris base, Boric acid and EDTA) for 5 minutes and then submerged in TBE buffer in a horizontal electrophoresis apparatus. One volt/cm was applied for 10 minutes. Each experiment was conducted using as a positive control SAF previously treated with 100 µM of hydrogen peroxide for 10 minutes at 2°C–8°C, as described in the manufacturer kit. Then samples were dried, stained with SYBR green and scored using a fluorescent microscope (Zeiss, Germany) connected to a digital camera (Sony, Japan). Obtained images were analyzed using the Image J free software (NIH, USA).

Chip-seq assay:

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) assay was performed using the EZ-Magna ChIP kit (Millipore, Milan, Italy) according to the manufacturer's instructions with minor modification. pPrm1-RFP transfected cells were grown for 16-18h hours then harvested. Protein/DNA complexes were cross-linked with 0.5% formaldehyde for 10 minutes at RT. Cells were lysed with Lysis buffer according to the manufacturer's instructions and afterwards sonicated in nuclear buffer (six 30 sec pulses, power setting 10 and six 30 sec pulses, power setting 15 in ice with 50 seconds rest between pulses; Bandelin Sonopuls). Soluble chromatin was immunoprecipitated with anti-RFP antibody (Evrogen, Milan, Italy) directly conjugated with Magnetic Protein A beads. DNA–protein immune complexes were eluted and reverse cross-linked, and DNA was extracted using a spin filter column. DNA obtained from pRFP (negative control), pPR1-RFP (experimental sample) and input (sonicated DNA, reaction CTR) were subjected to ChIP sequencing.

ChIP sequencing

Next generation sequencing experiments, comprising samples quality control, were performed. Indexed libraries were prepared from 10 ng/ea ChIP DNA with TruSeq ChIP Sample Prep Kit (Illumina) according to the manufacturer’s instructions. Libraries were quantified using the Agilent 2100 Bioanalyzer (Agilent Technologies) and pooled such that each index-tagged sample was present in equimolar amounts, with final concentration of the pooled samples of 2nM. The pooled samples were subject to cluster generation and sequencing using an Illumina HiSeq 2500 System (Illumina) in a 1x50 single-end format at a final concentration of 8pmol. The raw sequence files

generated (.fastq files) underwent quality control analysis using FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/).

Raw reads were checked for quality with FastQC v0.11.3 (www.bioinformatics.babraham.ac.uk/projects/fastqc/) and then trimming and removal of adapters were performed with Trimmomatic v0.33 (Bolger, et al., 2014). Trimmomatic: A flexible trimmer for Illumina Sequence Data. *Bioinformatics*, btu170.), minimum PHRED-quality and minimum length was set to 35 and 25nt, respectively. The obtained reads were then mapped against the *Ovis aries* genome (v3.1) with STAR v2.4.0j (A. Dobin et al, Bioinformatics 2012; doi: 10.1093/bioinformatics/bts635). Multiple mapping reads and duplicated reads were then removed from the mapping files with samtools (v0.1.19-44428cd) and Picard Tools 1.118 (http://picard.sourceforge.net). Peak calling was performed with Homer v4.7 (Heintz et al., 2010) separately for each immunoprecipitation using the data coming from the sonicated DNA as input. For peak calling the option 'histone' of Homer was set, the option –minDist was set to 3000. Distribution of coverage was calculated by using bedtools v2.17.0 (BEDTools: a flexible suite of utilities for comparing genomic features, Aaron R. Quinlan, and Ira M. Hall, Bioinformatics, 2010, 26 (6): 841-842. doi: 10.1093/bioinformatics/btq033) merging the results of the two immunoprecipitations, plots were generated with gnuplot after normalizing each dataset for the number of mapped reads.

Nuclear transfer

Methods of in vitro embryo production were adapted from those previously described (Ptak et al., 2002). Oocytes were matured in vitro in a humidified atmosphere of 5% CO_2/air at 39°C for 24 h. Oocytes were incubated in Hepes-buffered TCM-199 medium containing 4 mg/ml BSA, 7.5 mg/ml Cytochalasin B and 5 mg/ml Hoechst 33342 in an incubator for 15 minutes. Oocytes manipulation was carried out with a piezo-driven enucleation/injection pipette (PiezoXpert, Eppendorf). Enucleation was carried out in Hepes-buffered TCM-199 medium with 0.4% (w/v) BSA and

Cytochalasin B with a Narishighe micromanipulator fitted to a Nikon Eclipse inverted microscope. No DNA vital dyes/UV irradiation was used to locate the chromosomes in the oocytes, but a blind aspiration of the cytoplasm surrounding the first polar body was conducted, and enucleation was confirmed later by Hoechst staining and UV irradiation of the aspired cytoplasmic fragments (Iuso et al., 2014). Enucleate oocytes were allowed to recover from the Cytochalasin B treatment and then directly injected with a nucleus, either from CTR or a Prm1-RFP fibroblasts suspended in PBS with 6% Polyvinylpyrrolidone (Sigma). Reconstructed oocytes were activated in Hepes-buffered TCM-199 medium containing 5 mg/ml Ionomycin for 5 minutes and then incubated in SOF medium plus antibiotics and 0.8% BSA containing 10 mM Dimethylaminopurine and 7.5 mg/ml Cytochalasin B for 3–5 hours and cultured for 10-12 hours in SOF enriched with 1% (v:v) minimum essential medium (MEM) nonessential amino acids (Gibco, Milan, Italy), 2% (v:v) basal medium Eagle (BME) essential amino acids, 1 mM glutamine, and 8 mg/ml BSA covered with mineral oil pre-washed in SOF. Cultures were checked for embryonic development every 24 hours till day 7 post activation.

Supplemental References

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2. Heinz, S., Benner, C., Spann, N., Bertolino, E., Lin, Y.C., Laslo, P., Cheng, J.X., Murre, C., Singh, H., Glass, C.K. (2010). Simple combinations of lineage-determining transcription factors prime cis-regulatory elements required for macrophage and B cell identities. Mol Cell. 38: 576-89.
3. Ptak, G., Clinton, M., Tischner, M., Barboni, B., Mattioli., M. & Loi P. Improving delivery and offspring viability of in vitro-produced and cloned sheep embryos. Biol Reprod. 67, 1719-1725 (2002).

4. Iuso, D., Czernik, M., Zacchini, F., Ptak, G. & Loi, P. A simplified approach for oocyte enucleation in mammalian cloning. Cell Repr. 15(6), 490-494 (2013).