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CTR9/PAF1c regulates molecular lineage identity, histone H3K36 trimethylation and genomic imprinting during preimplantation development

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ABSTRACT

Genome-wide epigenetic reprogramming is required for successful preimplantation development. Inappropriate or deficient chromatin regulation can result in defective lineage specification and loss of genomic imprinting, compromising normal development. Here we report that two members of the RNA polymerase II associated factor, homolog (*Saccharomyces cerevisiae*) complex (PAF1 complex) components, *Ctr9* and *Rtf1*, are required during mammalian preimplantation development. We demonstrate that *Ctr9*-deficient embryos fail to correctly specify lineages at the blastocyst stage. Expression of some lineage specific factors is markedly reduced in *Ctr9* knockdown embryos, including *Eomes*, *Elf5* and *Sox2*, while others are inappropriately expressed (*Oct4*, *Nanog*, *Gata6, Fgf4 and Sox17*). We also show that several imprinted genes (*Mest*, *Peg3, Snrpn* and *Meg3*) are aberrantly expressed although allele specific DNA methylation is not altered. We document a loss of histone H3 lysine 36 trimethylation (H3K36me3) in *Ctr9*-deficient embryos and confirm that knockdown of either *Setd2* or *Rtf1* results in similar phenotypes. These findings show that the PAF1 complex is required for mammalian development, likely through regulation of H3K36me3, and indicate functional conservation of the PAF1 complex from yeast to mammals *in vivo*.

Introduction

Fusion of the highly differentiated mammalian oocyte and sperm results in the formation of a totipotent zygote that initiates embryogenesis. During preimplantation development, critical genome-wide epigenetic reprograming occurs to ensure successful embryonic development, including maintenance of genomic imprinting and establishment of pluripotency (reviewed in (Hemberger et al., 2009; Surani et al., 2007)).

Through a phenotype-driven RNAi screen, we identified mouse *Ctr9*, PAF1/RNA polymerase II complex component, homolog (*S. cerevisiae*) *(Ctr9)* as being required for developmental and epigenetic events during preimplantation development. CTR9 is a component of the PAF1 complex (PAF1c), which consists of the 5 core proteins PAF1, RTF1, CTR9, CDC73 and LEO1. The PAF1c was first identified in yeast where it has been shown to prevent transcriptional activation and regulate cell cycle (Koch et al., 1999). The yeast complex is also required for COMPASS-mediated H3K4 methylation and Dot1-mediated H3K79 methylation (Krogan et al., 2003a) as well as appropriate polyadenylation of some transcripts (Penheiter et al., 2005). Additionally, maintenance of histone H3K4me3 and H3K36me3 have been shown to require functional PAF1c in both yeast and human cells (reviewed in (Jaehning, 2010)). Adding to the challenges in understanding the biological role of PAF1c, the complex can also recruit CHD1, an ATP-dependent chromatin-remodeling enzyme.

Deletion of *Paf1* and *Ctr9* in yeast leads to aberrant cell growth and cell cycle control (Koch et al., 1999). PAF1c associates with RNA pol II at coding regions during transcription elongation and has been shown to promote phosphorylation of the RNA pol II C-terminal domain (Nordick et al., 2008). Furthermore, the PAF1c is crucial for appropriate transcription termination and 3′ mRNA end formation (Nordick et al., 2008). Currently, the mechanistic role of PAF1c during mammalian development *in vivo* remains largely unexplored.

Recently, both *Ctr9* and *Rtf9* were identified in an RNAi screen in embryonic stem cells (ESCs) as regulators of pluripotency (Ding et al., 2009). Ding et al. showed in ESCs that PAF1c components bind to the promoters of *Oct4*, *Nanog* and *Sox2*, which are essential to maintain ES identity, and that knockdown (KD) of *Ctr9* or *Rtf1* resulted in premature differentiation – specifically to endoderm *in vitro*. One member of PAF1c, *Cdc73* (also called Parafibromin and *Hrpt2*), has been knocked out in the mouse and results in peri-implantation lethality as well as lethality when conditionally

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deleted in adult animals (Wang et al., 2008). Additionally, components of PAF1c have been shown to be required for heart and neural crest development in zebrafish (Langenbacher et al., 2011), developmental Hedgehog, Notch and Wnt signaling in flies (Bray et al., 2005; Mosimann et al., 2009; Tenney et al., 2006), as well as immune response and cancer progression in human cell lines (reviewed in (Tomson and Arndt, 2013)).

Here, we show that *Ctr9* and *Rtf1* are present in morula and blastocyst stage embryos and are necessary for normal preimplantation lineage identity and function. *Ctr9*-deficient embryos fail to form trophoblast outgrowths, and the differentiation potential of the ICM is suppressed. Some key lineage specific factors are reduced in the absence of *Ctr9*, including *Eomes*, *Elf5* and *Sox2*, while others are inappropriately expressed (*Oct4*, *Nanog*, *Gata6, Fgf4 and Sox17)*. Importantly, H3K36me3 was reduced in *Ctr9*-deficient embryos, indicating functional conservation of PAF1c from yeast to mammals *in vivo*.

Materials and methods

Production and culture of embryos

Superovulation of B6D2 F1 female mice (8 to10 weeks old) was performed using 10 IU PMSG (Sigma) followed by 10 IU hCG (Sigma) 46–48 h later. At 20–22 h post-hCG treatment, zygotes were collected from B6D2 F1 female mice mated to B6D2 F1 males. For allele specific analysis, widely derived mouse strains were used to distinguish parental alleles by transcribed SNPs – B6D2F1 dams were mated to PWD (mus. Molossinus) stud males. Hyaluronidase (ICN Pharmaceuticals, Costa Mesa, CA, USA) was used to remove cumulus cells. Zygotes were cultured in KSOM at 37 °C/5% CO_2/5% O_2 balanced in N_2 in a mixed gas incubator. Use of vertebrate animals for embryo production was in accordance with the University of Massachusetts IACUC.

Outgrowth assay

After removal of zona pellucida, blastocysts were cultured individually in 10% DMEM in wells coated with 0.1% gelatin (Sigma). For ICM isolation, blastocysts were treated with anti-mouse antiserum, washed and exposed to guinea pig complement sequentially as described by (Solter and Knowles, 1975). The isolated ICMs were cultured individually in wells plated with 0.1% fibronectin. Outgrowths (intact blastocysts or isolated ICMs) were cultered at 37 °C/5% CO_2 in air and were observed for three days.

Double-stranded RNA (dsRNA) preparation

DNA templates for T7-RNA polymerase mediated dsRNA production were amplified from genomic DNA or preimplantation embryo cDNA using primers that contained the T7 binding sequences followed by gene specific sequences as follows: dsGfp Forward: TAATACGACTCACTATAGGGCACATGAAGCAGCACGACTT and Reverse: TAATACGACTCACTATAGGGTGCTCAGGTAGTGGTTGTCG,dsCtr9-1 Forward: TAATACGACTCACTATAGGGCCATTTGGCAAACCACTTTT and Reverse:TAATACGACTCACTATAGGGAAGGGCACCCTGTATGTCAG,dsCtr9-2Forward:TAATACGACTCACTATAGGGCCATGGCCAGAGATAAAGGAandReverse:TAATACGACTCACTATAGGGCGAAAACATCACGAGCTTCA,dsRtf1Forward:TAATACGACTCACTATAGGGCATGAAGAAGCAAGCCAACA and Reverse: TAATACGACTCACTATAGGGAACGCCGTTCTTTATTGTGG,dsSetd2Forward:TAATACGACTCACTATAGGGGATGGCTTGCACTCATCAGA and Reverse: TAATACGACTCACTATAGGGTCTCCAACTCTTGCCTTCGT. dsRNA apmicons were BLASTed against the mouse genome to ensure the target sequence had less than 19-mer homology with any non-target transcripts. PCR products were purified by gel extraction (Qiagen spin column-Qiagen # 28106). *In vitro* transcription was performed using T7 MEGAscript Kit (Ambion product # AM1334) and 0.5 μl of TURBO RNase-free DNase was added to each 10 μl reaction to remove the DNA template. dsRNA was treated with NucAway Spin Columns (Ambion product # AM10070) to recover the dsRNA while removing salts and unincorporated nucleotides. The dsRNA was then extracted with phenol: chloroform and precipitated with 70% ethanol and resuspended in RNase free water. The quality of dsRNA was confirmed by electrophoresis (after *in vitro* transcription as well as after precipitation). The concentration of dsRNA was measured by Nanodrop and diluted to 1 μg/μl and stored at −80 °C.

Microinjection

dsRNA was microinjected into the cytoplasm of zygote using a Piezo-drill (Prime Tech, Japan) and Eppendorf transferman micromanipulators. 1 μg/μl dsRNA were loaded into microinjection pipette and constant flow was adjusted to allow successful microinjection. Approximately, 5–10 pl dsRNA was injected into the cytoplasm of each embryo. For dsOct4 rescue experiment, 25 ng/μl dsOct4 was co-microinjected with dsCtr9.

RNA extraction, PCR and real-time PCR

Total RNA from embryos ($n=5$–10/pool) was extracted using the Roche High Pure isolation kit (Roche product # 1828665). cDNA synthesis was performed using M-MLV Reverse Transcriptase (product # M1701). To quantify gene expression differences between KD and control groups, real-time PCR was performed on a Stratagene MX3005p using Applied Biosystems Gene Expression Assays and Quanta Supermix (product # 95078) probe based reactions. All qPCR reactions included *Gapdh* Vic-labeled multiplex control. One embryo equivalent of cDNA was used for each real-time PCR reaction with a minimum of three replicates for all results shown. Taqman Gene Expression Assays from Applied Biosystems used: *Ctr9*, Mm00493862_m1; *Rtf1*, Mm01324605_m1; *Setd2*, Mm01250225_m1; *Elf5*, Mm00468732_m1; *Eomes*, Mm01351985_m1; *Mest*, Mm00485003_m1; *Peg3*, Mm01337379_m1; *Snrpn*, Mm02391920_g1; *Cdx2*, Mm01212280_m1; *Oct4*, Mm00656129_gH; *Nanog*, Mm01617761_g1; *Sox2*, Mm00488369_s1. Unless otherwise stated, quantification was normalized to *Gapdh* (ABI, 4352339E-0806018) mRNA.

Immunofluorescence

Preimplantation embryos were fixed with 4% paraformaldehyde in PBS for 25 min at room temperature, permeabilized with 0.2% Triton X-100 for 25 min, then blocked in 10% FBS/0.1% Triton X-100/PBS for 1 h after 3 times washing in 0.1% Triton X-100 PBS, and incubated with antibodies for 1 h at room temperature or overnight at 4 °C followed by incubation with Alexa Flour secondary antibodies 488, 543, 647 (Invitrogen) at 37 °C for 1 h. DNA was stained with DAPI and samples were mounted and observed with the Nikon Eclipse TE-2000-S microscope (Nikon). Identical image capture settings were used for embryos in experiment. Antibodies used are listed as follows: Immunogen (Vendor, Catalog #, Dilution, and Species). CDH1 (Abcam, Ab53033, 1:200, Rabbit); CDX2 (Biogenex, AM392-5 M, 1:200, Mouse); CTR9 (Abcam, Ab84487, 1:200, Rabbit); ELF5 (Santa Cruz, sc9645, 1:100, Goat); EOMES (Abcam, Ab23345, 1:200, Rabbit); GATA6 (R&D Systems, AF1700, 1:200, Goat); H3K36me3 (Abcam, Ab9050, 1:200, Rabbit); H3K4me3 (Abcam, Ab8580, 1:200, Rabbit); H3K9me3 (Abcam, Ab8988, 1:200 Rabbit); NANOG (CosmoBio, RCAB0002P-F, 1:200, Rabbit); OCT4 (Santa Cruz, sc5279, 1:200, Mouse); SOX17 (R&D Systems, NL1924R, 1:300, Goat); and SOX2 (Santa Cruz, sc17320, 1:200, Goat). For IF experiments, we