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The Role of PTIP in Maintaining Embryonic Stem Cell Pluripotency

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ABSTRACT

Pax transactivation domain-interacting protein (PTIP) is a ubiquitously expressed, nuclear protein that is part of a histone H3K4 methyltransferase complex and is essential for embryonic development. Methylation of H3K4 is an epigenetic mark found on many critical developmental regulatory genes in embryonic stem (ES) cells and, together with H3K27 methylation, constitutes a bivalent epigenetic signature. To address the function of PTIP in ES cells, we generated ES cell lines from a floxed *ptip* allele and deleted PTIP function with Cre recombinase. The *ptip*$^{-/-}$ ES cell lines exhibited a high degree of spontaneous differentiation to trophectoderm and a loss of pluripotency. Reduced levels of Oct4 expression and H3K4 methylation were observed. Upon differentiation, *ptip*$^{-/-}$ embryoid bodies showed reduced levels of marker gene expression for all three primary germ layers. These results suggest that the maintenance of H3K4 methylation is essential and requires PTIP function during the in vitro propagation of pluripotent ES cells. STEM CELLS *2009;27: 1516–1523*

Disclosure of potential conflicts of interest is found at the end of this article.

INTRODUCTION

Embryonic stem (ES) cells can remain pluripotent in culture and contribute to all tissues including the germ line when reintroduced into a developing embryo. Pluripotency requires the continued expression of Oct4 and Nanog, two transcriptional factors that regulate a network of genes in ES cells [1]. Differentiation into specialized cell types was thought to be stable and irreversible. However, the reintroduction of a limited number of genes, including *oct4*, *sox2*, *c-myc*, and *klf4*, and subsequent selection for *nanog* expression can dedifferentiate somatic cells into an ES cell–like state [2, 3]. Differentiation and loss of pluripotency may be driven, at least in part, by epigenetic modifications of chromatin, including histone methylation at specific lysine residues. Consistent with the idea that ES cell chromatin is epigenetically plastic, the patterns of histone methylation at key regulatory loci in ES cells show low levels of both active and inactive epigenetic marks that are resolved upon differentiation into fully active or fully repressed marks depending upon cell lineage [4, 5].

The mammalian homologues of the *Drosophila* Trithorax and Polycomb group genes encode the histone modification machinery that specifies active or inactive regions of the genome. Genetic studies in mice reveal an essential role for histone methyltransferases and their associated factors in early embryonic development, as cells assume a more differentiated fate and lose pluripotency [6–11]. However, the roles of histone methyltransferase complexes in maintaining growth and pluripotency of cultured ES cells have not been well studied.

Methylation of histone H3 at lysine four (H3K4) is a key modification that correlates with gene expression and is thought to promote assembly of nucleosome remodeling complexes required for transcription, elongation, and splicing [12, 13]. In ES cells, H3K4 trimethylation is present at the transcription initiation sites of many genes and is coupled to RNA polymerase occupancy, even if in most cases transcriptional elongation and mRNA expression do not progress [14]. The mammalian homologues of Trithorax are the MLL family of H3K4 histone methyltransferases, which copurify with the accessory proteins WDR5, RBBP5, and ASH2L, similar to the yeast Set1 COMPAS methyltransferase complex [15, 16]. The BRCA1 C-terminal domain–containing protein Pax transactivation domain-interacting protein (PTIP) is a novel component of the MLL2 methyltransferase complex [17, 18] and is essential for embryonic development postgastrulation [19]. PTIP interacts with the developmental regulatory transcription factor Pax2 and promotes assembly of the MLL2 histone H3K4 methyltransferase complex at a Pax2 DNA-binding site [20]. However, PTIP is likely to interact with other DNA-binding proteins to impact patterns of histone modification at many loci, as both germline null and conditional mutants show reduced levels of H3K4 di- and trimethylation in affected tissues. These data suggest that PTIP is critical for linking the MLL2 and MLL3 complexes to specific DNA-binding transcription factors during differentiation, such that H3K4 methylation is regulated in a locus- and tissue-specific manner.

The developmental defects observed in *ptip*$^{-/-}$ homozygous embryos are evident at the time of gastrulation and result in a disorganized mass of poorly differentiated cells. Many *ptip*$^{-/-}$ nuclei exhibit free DNA ends, are stuck in the

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cell cycle, and exhibit reduced levels of global H3K4 methylation [19, 20]. Even at earlier stages, blastocyst explants from embryonic day (E)3.5 embryos showed clear inhibition of inner cell mass (ICM) proliferation in $ptip^{-/-}$ embryos. However, these experiments did not assess the role of PTIP in maintaining ES cell pluripotency. If H3K4 methylation is important for maintaining potency then the loss of PTIP may affect global levels and lead to a reduction in the differentiation potential of stem cells. In order to test this hypothesis, we derived ES cell lines from mice carrying one conditional floxed *ptip* allele and one null allele ($ptip^{fl/-}$) and generated a PTIP loss-of-function mutation in ES cell lines with Cre recombinase under conditions that should retain pluripotency. The ES cells lacking PTIP underwent spontaneous differentiation despite the optimal medium, with feeder cells and recombinant leukemia inhibitory factor (rLIF). The loss of ES cell pluripotency was assayed by real-time quantitative reverse transcription-polymerase chain reaction (qRT-PCR), Western blotting, alkaline phosphatase staining, trophectoderm-specific marker 1 (TROMA-1) immunostaining, and chromatin immunoprecipitation (ChIP). The data suggest a crucial role for PTIP and H3K4 methylation in maintaining the undifferentiated state and pluripotency of ES cells in culture.

Materials and Methods

Generation of PTIP-Deficient Mouse ES Cells

To address the functions of PTIP in ES cells, *ptip* floxed mice were generated as described previously [21]. Mice carrying one floxed allele were intercrossed to generate homozygous $ptip^{fl/fl}$ mice that are viable and fertile. Three- to five-week-old females $ptip^{fl/fl}$ mice were superovulated by i.p. injection with pregnant mare serum gonadotropin and human chorionic gonadotropin 2 days before and on the day of mating with $ptip^{fl/-}$ mice. Three days later, blastocysts were flushed out of the uterine horns and cultured in ES cell growth medium as described elsewhere [22]. The $ptip^{fl/-}$ or $ptip^{fl/fl}$ ES cells were selected for infection with Cre-expressing adenovirus (Ad-Cre). The ES cells were split onto mouse embryo fibroblast (MEF) feeders and infected with Ad-Cre or adenovirus alone (Ad) at 1,000:1 multiplicity of infection (MOI) for 4 hours, and fresh medium was added after removal of the medium with the virus. After 2 days, colonies were picked and transferred to individual wells of a 96-well plate with MEFs and rLIF. After two additional days, ES cells were split into 24-well plates. Genotyping was done at passage 4–6 to see if *ptip* was deleted by the Ad-Cre. At that passage, two clones were identified as PTIP-deficient ES cells and were expanded in six-well plates for an additional two to four passages before being used for making embryoid body (EB) and other assays.

The $ptip^{fl/-}$ ES cells were also used for generating proteins, RNAs, and chromatin from Ad-Cre–infected cells without clonal selection. After trypsinization, 1×10^6 cells were plated onto gelatin-coated 60-mm plates with rLIF and infected with adenoviral vectors at an MOI of 500. Cells were harvested after 4 days and deletion of PTIP was monitored by Western blotting.

Immunocytochemistry and Alkaline Phosphatase Staining

ES cells were seeded onto glass coverslips and allowed to grow for up to 3 days. Growth medium was removed and the cells were fixed in 4% paraformaldehyde for 10 minutes. The primary antibodies used were anti-rabbit Oct4 (Santa Cruz Biotechnology Inc., Santa Cruz, CA, http://www.scbt.com) or mouse anti-TROMA-1, diluted 1:100 each in 0.1% Tween 20 in phosphate-buffered saline (PBST) plus 2% goat serum and applied for 1 hour at room temperature. Secondary antibody, anti-rabbit IgG-fluorescein isothiocyanate conjugated or anti-mouse IgG-tetramethylrhodamine isothiocyanate conjugated, diluted 1:200 in PBST plus 2% goat serum, was applied for 1 hour at room temperature. Cells were further counterstained with 4′,6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA, http://www.vectorlabs.com) and imaged on a Nikon ES800 fluorescent scope (Nikon Instruments, Inc., Melville, NY, http://www.nikoninstruments.com) with a SPOT digital camera (Diagnostic Instruments Inc., Sterling Heights, MI, http://www.diaginc.com).

Alkaline phosphatase substrate solution was prepared using Sigma Fast fast red tablet sets (F-4523; Sigma-Aldrich, St. Louis, http://www.sigmaaldrich.com) as per the manufacturer's instructions. ES cells on glass coverslips were incubated in the solution in a 37°C incubator for 1 hour and monitored for the development of a red reaction product, indicating alkaline phosphatase activity.

Western Blotting

Cells were lysed in protein lysis buffer [23] and protein levels were quantified by the Bio-Rad colorometric assay (Bio-Rad, Hercules, CA, http://www.bio-rad.com). Histone proteins were acid extracted as described (Abcam, Cambridge, U.K., http://www.abcam.com). SDS-PAGE sample buffer was added and samples were boiled for 5 minutes. Samples were run on a 10% polyacrylamide gel, transferred to a polyvinylidene difluoride membrane (PerkinElmer Life and Analytical Sciences, Boston, http://www.perkinelmer.com), and blocked with 5% nonfat milk in Tris-buffered saline. Membranes were immunoblotted with anti-rabbit Oct-3/4, anti-mouse β-actin, anti-rabbit trimethyl H3K4 (Abcam), and mono-, di-, trimethyl H3K4 (Abcam) antibodies. For quantitation, secondary antibodies were coupled to fluorescent-conjugated secondary antibodies and a Li-Cor Odessey infrared imager (Li-Cor Biosciences, Lincoln, NE, http://www.licor.com) was used.

Real-Time RT-PCR

Total RNA extraction and the RT-PCR analysis were performed as described previously [22]. The real-time PCR primer pairs were as follows: Oct-4, AGCTGCTGAAGCAGAAGAGG and GGTTCTCATTGTTGTCGGCT; Hand-1, GGATGCACAAGCA GGTGAC and CACTGGTTTAGCTCCAGCG; vimentin, AACA CCCGCACCAAC and TGTCCCGCTCCACCTCGAC; enolase, TGCTAAGGCCCTTTTCTGTT and GACTAGGCACCCCTAT TCCA; α-fetoprotein, CACTGCTGCAACTCTTCGTA and CTTT GGACCCTCTTCTGTGA; Hnf-4, ACACGTCCCCATCTGAAG and CTTCCTTCTTCATGCCAG; Nkx2.5, AGCAACTTCGTGA ACTTTG and GATCCGGTCCTAGTGTGGA; GATA-4, GGTT CCCAGGCCTCTTGCAATGCGG and AGTGGCATTGCTGGA GTTACCGCTG; PTIP, CCGAAGTTCCAGAGGAGCTA and GTCCCCATCCTCGAAATGA; and glyceraldehyde-3-phosphate dehydrogenase, TCCGCCCCTTCTGCCGATG and CACGGA AGGCCATGCCAGTGA.

ChIP

ChIP was performed in triplicate according to published protocols from Upstate Biotech (Charlottesville, VA, http://www.upstate.com) with minor modifications. Cells were fixed for 10 minutes at 25°C with 1% formaldehyde in culture medium. Crosslinking was stopped by the addition of glycine to 0.125 M. Cells were washed twice with ice-cold PBS, scraped, and harvested by centrifugation. The cell pellet was washed in PBS, resuspended in cell lysis buffer (5 mM PIPES, pH 8.0, 85 mM KCl, 0.5% NP40, protease inhibitors), incubated at 4°C for 5 minutes, and centrifuged for 5 minutes at 3,000*g*. The nuclei were resuspended in nuclei lysis buffer (50 mM Tris-HCl, pH 8.1, 10 mM EDTA, 1% SDS, protease inhibitors) and were sonicated on ice with three 20-second pulses using a microtip probe sonicator (Branson Sonifier 250, Branson Ultrasonics Inc., Danbury, CT, http://www.sonifier.com) with the output control set to 1. Sonicated lysates were clarified by centrifugation at 4°C for 15 minutes. Twenty micrograms of chromatin was diluted in IP dilution buffer (0.01% SDS, 1.1% Triton-X100, 1.2 mM EDTA, 16.7 mM Tris-HCl, pH 8.1, 167 mM NaCl) and precleared with 80 μl protein A-agarose

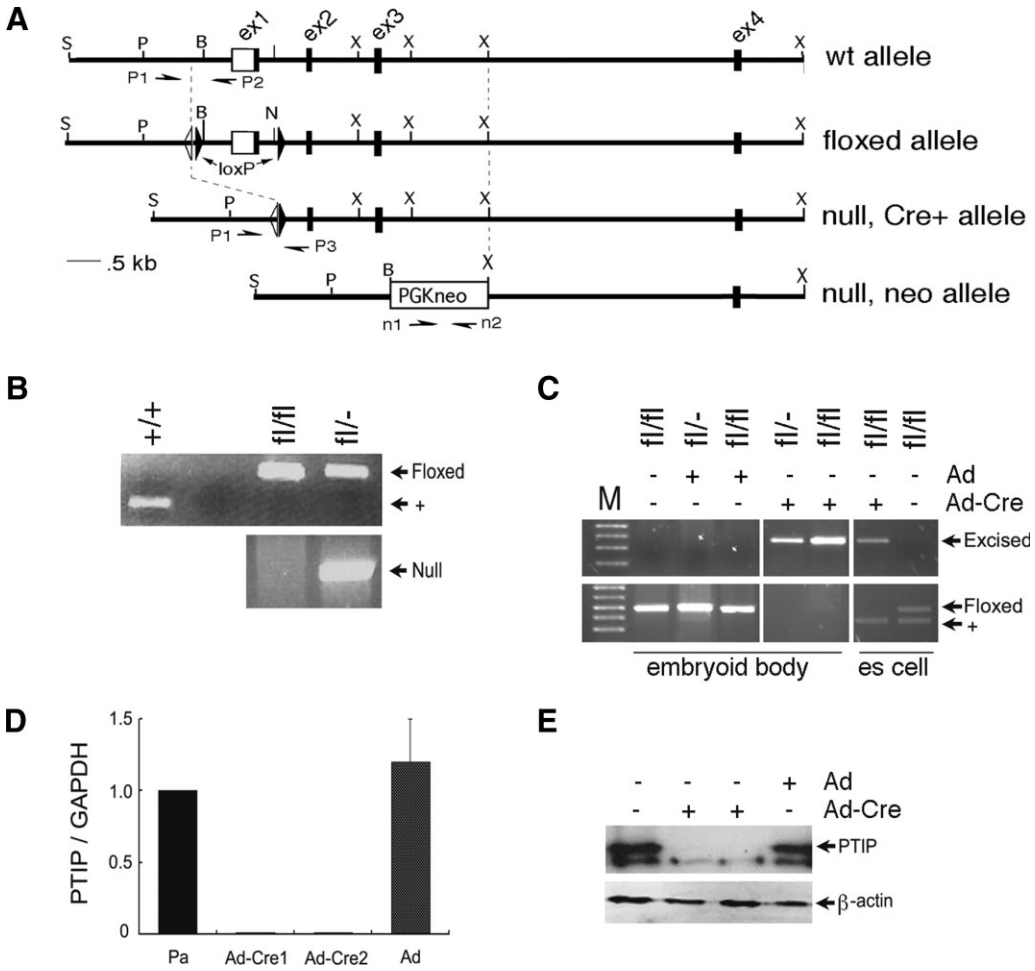

Figure 1. Generation of *ptip*$^{-/-}$ ES cells. PCR genotyping of ES cells with allele-specific primer pairs. (**A**): Schematic of the different alleles used in this study. The floxed allele contains loxP sites flanking exon 1 (ex1); a single flip recombinase site is also present (open triangle). The germline null allele deletes exons 1–3 and still contains PGK-neo, as described previously. Primers P1 and P2 were used to detect the wt and floxed alleles, primers P1 and P3 detect the Cre excised allele, and primers n1 and n2 detect the neo-containing null allele. (**B**): wt and ES cell lines derived from a *ptip*$^{fl/fl}$ × *ptip*$^{fl/-}$ mating were genotyped with allele-specific primer pairs before Cre-mediated excision. The top panel indicates the wt (+) and floxed alleles. The bottom panel shows the germline null allele. (**C**): Genotype of ES cells after infection with Ad-Cre. As indicated, cells were cultured as embryoid bodies, without feeders, or as ES cells on feeders and LIF. Note, the wt allele is a result of the feeder cells. (**D**): Relative mRNA expression determined by real-time quantitative PCR. Parental *ptip*$^{fl/-}$ ES cells (Pa), *ptip*$^{-/-}$ derivatives (Ad-Cre1, Ad-Cre2), and parental cells infected with Ad only are shown. (**E**): PTIP protein expression as determined by Western blotting from *ptip*$^{fl/-}$ ES cells infected with the control Ad or Ad-Cre as indicated. Abbreviations: Ad, adenovirus; Ad-Cre, Cre-expressing adenovirus; ES, embryonic stem; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; LIF, leukemia inhibitory factor; PCR, polymerase chain reaction; PTIP, Pax transactivation domain-interacting protein; wt, wild-type.

or protein G-agarose (Upstate). The precleared chromatin preparation was then precipitated with 2–5 μg of antibodies against trimethyl H3K4 (Abcam) or mono-, di-, trimethyl H3K4 (Upstate). After overnight incubation at 4°C, 60 μl protein A-agarose or protein G-agarose was added and the incubation was continued for 1 hour. The beads were sequentially washed two times in IP dilution buffer, two times in TSE-500 wash buffer (0.1% SDS, 1% Triton X-100, 2 nM EDTA, 20 mM Tris-HCl, pH 8.1, 500 mM NaCl), two times in LiCl buffer (100 mM Tris-HCl, pH 8.1, 500 mM LiCl, 1% NP-40, 1% sodium deoxycholate), and finally two times in Tris-EDTA. Bound complexes were eluted by vortexing beads twice for 15 minutes at 25°C in 250 μl of elution buffer (50 mM sodium bicarbonate and 1% SDS). Five molar NaCl was added to a final concentration of 0.2 M to the pooled eluates and crosslinks were reversed by incubating samples at 65°C overnight. The samples were digested with PK for 1 hour at 56°C and phenol-chloroform extracted. The precipitated DNA was reconstituted in sterile water, and real-time PCR quantitation of precipitated genomic DNA relative to inputs was performed in triplicate using IQ SYBR Green mastermix (Bio-Rad) in an iCycler (Bio-Rad). *oct4, sox2, and pax3* genomic sequences were analyzed by real-time PCR (Bio-Rad). The data are represented as mean ± one standard error of the mean of two independent experiments. Primer pairs were as follows: Oct4-F1, gtgaggtgtccggtgacccaaggcag (−235) and Oct4-R1, ggcgagcgctatctgcctgtgtc (−94); Oct4-F3, ctgtaaggacaggccgagag (−308) and Oct4-R3, caggaggccttcattttcaa (−170); Sox2-F2, tgccagcttcctgaaatact (−442) and Sox2-R2, gttcgaaggaagtgggtaaa (−265); Sox2-F3, gcattttagccacaaaggtt (−1,453) and Sox2-R3, attttagccgatctccccatt (1,262); and Pax3-F1, gggcggatatagcaaggtttcc (−370) and Pax3-B1, cctgtccctctacatgagattcagc (−270).

Results

The function of PTIP in the epigenetic maintenance of ES cell cultures was addressed by deleting PTIP in ES cell lines derived

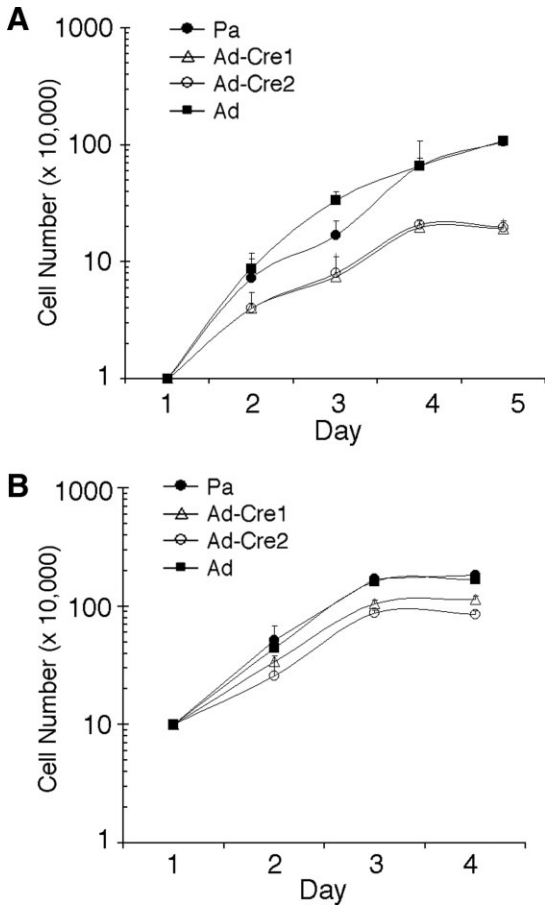

Figure 2. Growth curves of ES cells cultured on MEFs. (**A**): ES cells were seeded at 10^4 cells/ml and counted daily thereafter. (**B**): ES cells were seeded at 10^5 cells/ml and counted daily, reaching saturation within 3 days. Parental ES $ptip^{fl/-}$ cell lines (Pa) or $ptip^{fl/-}$ ES cells infected with control Ad were compared with $ptip^{fl/-}$ ES cells infected with Ad-Cre (Ad-Cre1, Ad-Cre2). Abbreviations: Ad, adenovirus; Ad-Cre, Cre-expressing adenovirus; ES, embryonic stem; MEF, mouse embryo fibroblast.

from $ptip^{fl/-}$ or $ptip^{fl/fl}$ mouse blastocysts using Cre recombinase–mediated excision. We were able to derive stable, parental ES cell lines from a cross of $ptip^{fl/fl}$ and $ptip^{fl/-}$ animals that were homozygous *fl/fl* or heterozygous *fl/−*. These cells were grown on MEFs with rLIF and maintained a pluripotent ES cell phenotype for many generations. For generating clonal lines, the $ptip^{fl/-}$ ES parental cell lines were infected with either Ad-Cre or Ad alone to delete exon 1 and the 5′ regulatory region of the *ptip* gene (Fig. 1). Two early-passage $ptip^{-/-}$ cell lines were established that deleted the *ptip* floxed allele (Ad-Cre1 and Ad-Cre2). At passage 8–10, neither cell line had detectable amounts of PTIP mRNA or protein, as measured by RT-PCR and Western blotting, respectively (Fig. 1C, 1D).

Our previous work with complete $ptip^{-/-}$ embryos showed a defect in proliferation in blastocyst outgrowth culture [19]. However, these outgrowths did not contain feeder cells or rLIF to maintain pluripotency. To determine if $ptip^{-/-}$ ES cells can grow under conditions that maintain pluripotency, cell proliferation was measured after seeding at either low (1×10^4/ml, Fig. 2A) or high (1×10^5/ml, Fig. 2B) density in rLIF and MEF feeder cells (Fig. 2). At low density, parental ES cells grew up to 1×10^6/ml by 5 days, whereas $ptip^{-/-}$ ES cells grew to no more than 3×10^5/ml. At high density, differences in growth rates were less pronounced, as $ptip^{-/-}$ ES cells grew to nearly 1×10^6/ml when seeded at high density.

Given that ES cells are self-renewing, $ptip^{-/-}$ cells might lose their self-renewal capacity when seeded at low density, but maintain self-renewal, at least in part, at higher densities. Self-renewal maintains the pluripotent state and suppresses spontaneous differentiation. Thus, cells were subjected to alkaline phosphatase staining, which stains only undifferentiated ES cell colonies (Fig. 3). Colonies from $ptip^{-/-}$ ES cells seeded at low density stained poorly for alkaline phosphase and were generally small with a more flattened phenotype (Fig. 3B–3D). At higher densities, $ptip^{-/-}$ ES cells stained positive for alkaline phosphatase but intensities were weaker and colony size was smaller than with wild-type ES cells (Fig. 3E–3H). These data suggest that $ptip^{-/-}$ ES cells undergo spontaneous differentiation even in the presence of MEFs and rLIF.

The maintenance of pluripotency in ES cells depends on the continued expression of the transcription factor Oct4. In

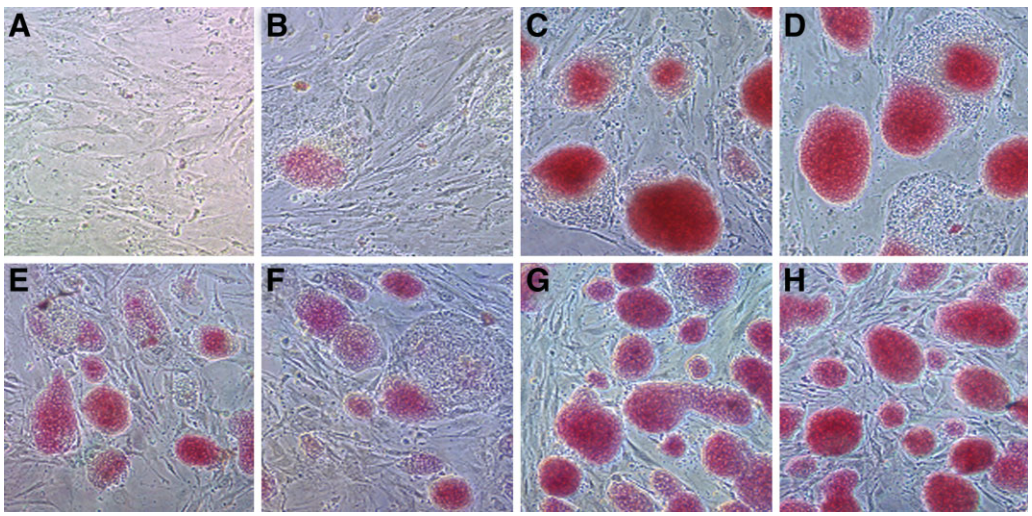

Figure 3. Alkaline phosphatase staining of PTIP-deficient ES cells. (**A–D**): Five days after seeding at 10^4 cells/ml. (**E–H**): Three days after seeding at 10^5 cells/ml. All cells were cultured with MEFs and LIF. (**A**): MEFs only. (**B**): $ptip^{fl/-}$ Ad-Cre1 line. (**C**): Parental line $ptip^{fl/-}$. (**D**): Parental $ptip^{fl/-}$ line infected with Ad only. (**E**): $ptip^{fl/-}$ Ad-Cre1 line. (**F**): $ptip^{fl/-}$ Ad-Cre2 line. (**G**): Parental $ptip^{fl/-}$ line. (**H**): Parental $ptip^{fl/-}$ line infected with Ad only. Abbreviations: Ad, adenovirus; Ad-Cre, Cre-expressing adenovirus; ES, embryonic stem; LIF, leukemia inhibitory factor; MEF, mouse embryo fibroblast; PTIP, Pax transactivation domain-interacting protein.

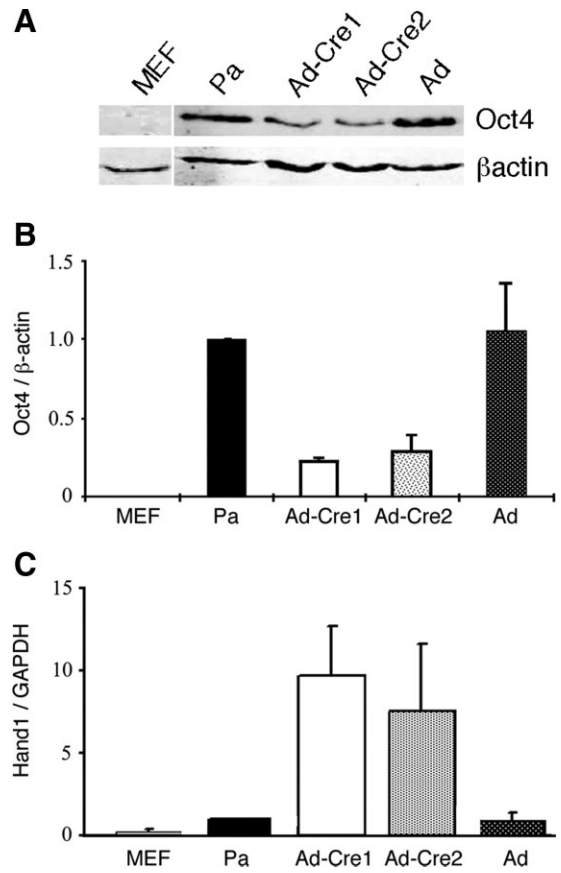

Figure 4. Oct4 and Hand1 expression in PTIP-deficient ES cells. (**A**): Western blotting of Oct4 and β-actin from parental ES cells and PTIP-deleted cells. (**B**): Quantitative protein measurements of Oct4 normalized to β-actin, using fluorescent-conjugated secondary antibodies and a LiCor reader. (**C**): Real-time RT-PCR of Hand1 normalized with GAPDH. Parental $ptip^{fl/-}$ ES line (Pa), control Ad-infected $ptip^{fl/-}$ ES cells (Ad), and $ptip^{fl/-}$ Ad-Cre–expressing cells (Ad-Cre1, Ad-Cre2) were compared. All data were produced from three independent experiments. Error bars are one standard deviation from the mean. Abbreviations: Ad, adenovirus; Ad-Cre, Cre-expressing adenovirus; ES, embryonic stem; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MEF, mouse embryo fibroblast; PTIP, Pax transactivation domain-interacting protein; RT-PCR, reverse transcription-polymerase chain reaction.

$ptip^{-/-}$ ES cell lines, Oct4 mRNA and protein levels are reduced, exhibiting three- to fourfold less protein (Fig. 4A, 4B). However, $ptip^{-/-}$ ES cells showed a marked seven- to ninefold greater expression level of the trophectodermal marker Hand1, as measured by real-time qRT-PCR (Fig. 4C). These data are consistent with the hypothesis that $ptip^{-/-}$ ES cells spontaneously differentiate into the trophectodermal lineage, perhaps in part by downregulating Oct4. This spontaneous loss of Oct4 may not affect the ability of PTIP-deficient cells to differentiate after removal of LIF, because Oct4 is generally downregulated upon formation of the three primary germ layers. To examine this directly, we cultured cells under conditions that favored EB formation and examined the cultures for gene expression over time. The EBs obtained from $PTIP^{-}$ cells were much smaller than the $PTIP^{+}$ EBs and expressed lower levels of differentiation markers (Fig. 5). Markers for all three germ layers were used, including ectoderm (vimentin and enolase), endoderm (α-fetoprotein and Hnf-4), and mesoderm (Nkx2.5). Given the lower levels of expression of all markers coupled with the greater expression of Hand1, it is likely that PTIP-deficient ES cells are unable to differentiate into the normal germ layers, again implying that they have lost much of their pluripotency and prefer the trophectodermal lineage.

Finally, we assessed this effect at the single-cell level by staining parental and $ptip^{-/-}$ ES cell colonies with antibodies against Oct4 and the trophectodermal marker TROMA-1 (Fig. 6A). Wild-type ES cells exhibited strong nuclear staining with anti-Oct4 and very little staining with anti-TROMA-1. Consistent with the Western blotting and RT-PCR data, $ptip^{-/-}$ ES cell colonies were heterogeneous, with some cells $Oct4^{+}$ and TROMA-1^{-}, yet many other cells TROMA-1^{+} and $Oct4^{-}$. Over 90% of $ptip^{-/-}$ ES cell colonies cultured at high density contained TROMA-1^{+} cells, whereas no wild-type ES cell colonies exhibited detectable staining with the same antibody (data not shown). Therefore, we conclude that a fraction of $ptip^{-/-}$ ES cells differentiated spontaneously into trophectodermal cells over time. This spontaneous differentiation affected the stability of the ES cell clones after multiple passages. By passage 12–15, the self-renewing capacity of the ES cell clones was limited, making long-term culture of the PTIP-null cells difficult.

Given the potential for clonal effects after Ad-Cre infection and clonal selection, we also examined the expression of ES cell markers in a population of $ptip^{fl/fl}$ ES cells that had been infected with Cre and control virus. Cells grown on feeders with rLIF were trypsinized and plated on gelatin-coated plates with rLIF only, infected with Cre or control virus, and harvested 96 hours postinfection. PTIP excision was monitored by Western blotting of lysates (Fig. 6B); expression of pluripotency markers was also assessed (Fig. 6B). The data indicate that the loss of Oct4 and Sox2 expression is coincident with PTIP excision in Cre-infected cells. These data are consistent with the changes in gene expression observed in clonal isolates after Cre-mediated excision at low passage numbers.

The PTIP protein is part of a histone H3, lysine four (H3K4) methyltransferase complex that includes the proteins MLL2, MLL3, ASH2L, WDR5, and RBBP5 [17, 18, 20]. In cell culture models, PTIP is necessary for the assembly of an H3K4 methyltransferase complex in response to binding of the developmental regulatory protein Pax2 to its DNA recognition site. Consistent with this model, $ptip^{-/-}$ embryonic tissues show lower global levels of di- and trimethylation at H3K4, but not at lysine 20 of histone H4. To test whether global levels of H3K4 methylation were affected, we did quantitative Western blotting of parental and $ptip^{-/-}$ ES cell histones after acid extraction from clonal cells at lower passages (Fig. 7A). Antibodies against trimethyl-H3K4 or mono-di-trimethyl-H3K4 showed a two- to threefold lower total methylation level than wild-type ES cells, implying that the mutant ES cells have altered histone H3K4 methylation patterns at many loci.

To examine whether the reduction in H3K4 methylation could occur at specific loci, chromatin immunoprecipitation experiments were performed using antibodies against methyl H3K4 and PCR primer pairs corresponding to putative promoter elements (Fig. 7B). Populations of Ad-Cre–infected ES cells were prepared without clonal selection, as assayed in Figure 6B. Because expression levels were lower in $ptip^{-/-}$ ES cells, the *oct4* and *sox2* promoters were examined, as well as the *pax3* gene, which encodes a developmental regulator. In $ptip^{-/-}$ ES cells, the *oct4* and *sox2* promoter sequences showed a lower level of H3K4 trimethylation than in wild-type ES cells. The *pax3* promoter did not show any measurable difference in H3K4 methylation, consistent with its low expression levels in both $PTIP^{+}$ and $PTIP^{-}$ cells (data not shown). Given the heterogeneous nature of the $ptip^{-/-}$ ES cells, this reduction in *oct4* H3K4 methylation is likely to

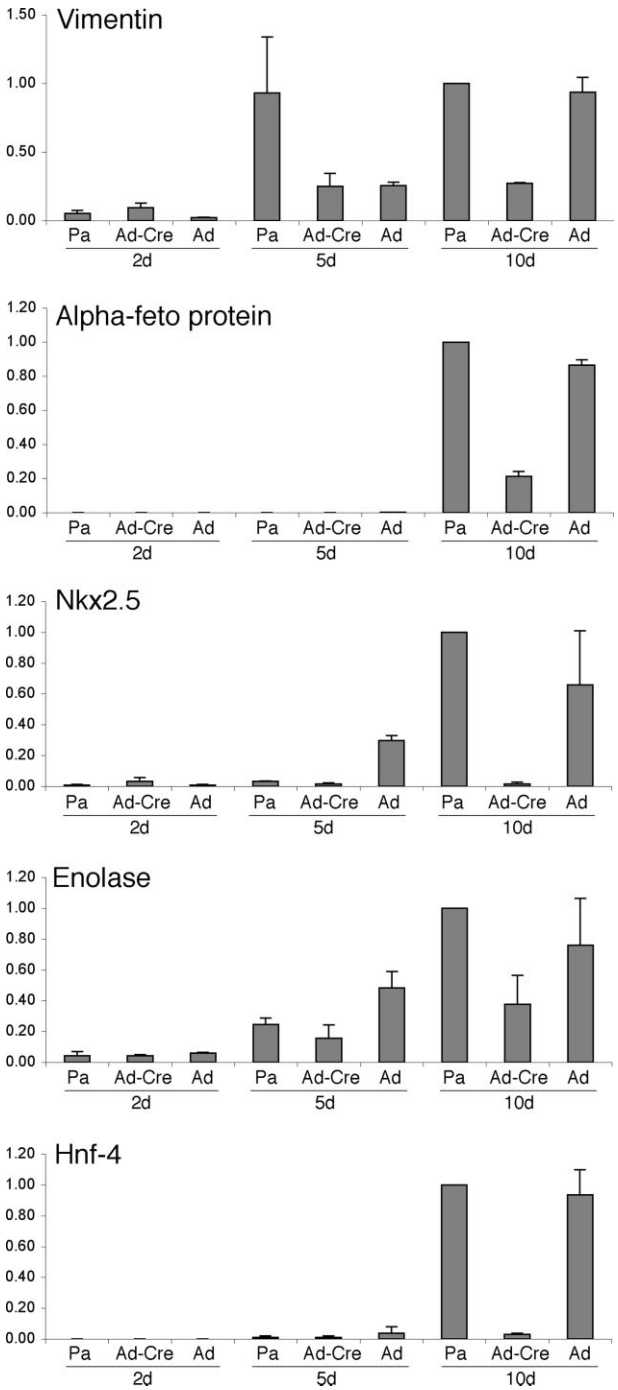

Figure 5. Expression of lineage markers in EBs. Real-time RT-PCR analysis of genes expressed in differentiating EBs over time. Markers for all three germ layers were used, including ectoderm (vimentin and enolase), endoderm (α-fetoprotein and Hnf4), and mesoderm (Nkx2.5). Parental ES $ptip^{fl/-}$ cells (Pa), Ad-Cre1–infected cells (Ad-Cre), and Ad-infected cells (Ad) were used to generate EBs in hanging drops that were then cultured for 2, 5, or 10 days in Petri dishes. Total RNA was isolated and RT-PCRs were normalized to GAPDH. Averages for three readings are shown; error bars represent one standard deviation from the mean. Abbreviations: Ad, adenovirus; Ad-Cre, Cre-expressing adenovirus; EB, embryoid body; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RT-PCR, reverse transcription-polymerase chain reaction.

reflect the proportion of cells that have turned down *oct4* completely.

Discussion

Pluripotent ES cells can maintain a differentiated state in culture indefinitely, whereas in vivo pluripotency is a transient state reserved for the ICM of the blastocyst, the epiblast cells of the postimplantation embryo, and the germ cells (for a review, see [24]). The maintenance of ES cell pluripotency requires a defined transcriptional regulatory circuit, consisting of Oct4, Sox2, and Nanog [25], and epigenetic factors such as Polycomb repressive complexes [26] and histone demethylases [27]. The chromatin signatures of critical regulatory genes in ES cells exhibit both H3K27 and H3K4 methylation [4, 5, 28], epigenetic marks that were thought to correlate with gene silencing and activation exclusively. Furthermore, acetylation at H3K9 and promoter occupancy by RNA polymerase II indicate that many nonexpressed genes in ES cells are in an accessible state, suggesting that polymerase elongation is the critical regulatory event [14]. Despite its prevalence at many loci, the necessity of H3K4 methylation complexes in ES cells has not been demonstrated. In this report, the function of PTIP was examined in cultured ES cells. Given that PTIP is an integral component of the MLL2 H3K4 methyltransferase complex [17, 18, 20], our data suggest that maintenance of H3K4 methylation is necessary for continued proliferation of pluripotent ES cells in culture.

During mouse development, deletion of the H3K4 methyltransferases ALL/MLL1 [9, 29] or MLL2 (which is homologous to human MLL4) [11] results in postgastrulation lethality, characterized by the misregulation of *hox* gene expression. Despite multiple cell autonomous defects, both $mll1^{-/-}$ and $mll2^{-/-}$ embryos progressed well past gastrulation, with axial patterning and germ layer differentiation easily recognizable at E8.5. In contrast, $ptip^{-/-}$ embryos showed significant developmental arrest by E7.5, resulting in a small and disorganized tissue mass by E8.5 [19, 20]. Mouse ES cells lacking either MLL1 [30] or MLL2 [11] have also been reported. For mouse *mll2* mutant ES cells, differentiation was delayed, but endodermal lineages and self-renewal seemed unaffected [31]. The severity of the $ptip^{-/-}$ phenotype suggests that the protein is interacting with more than just one H3K4 methyltransferase. The presence of MLL2 and/or MLL3 in the PTIP complex and the global reduction in H3K4 methylation observed in $ptip^{-/-}$ embryos support this hypothesis [17, 18, 20]. Thus, the available evidence suggests an earlier or more integral function for PTIP in early growth and differentiation than for any single H3K4 methyltransferase described to date.

The culture of ES cells is an artificial system that is able to suppress the transient nature of pluripotency. Through the use of feeder cells and LIF, factors such as Oct4, Nanog, and Sox2 are maintained indefinitely. Direct loss of Oct4 by gene targeting [32] or RNA interference [33] promotes trophoblast differentiation at the expense of ICM cells. Thus, the reduction in Oct4 protein over time could account for much of what is observed in the $ptip^{-/-}$ ES cells. This reduction in Oct4 and loss of pluripotency may be less critical in vivo because of maternal contributions and the limited number of cell divisions required of the pluripotent cells. Thus, the status of H3K4 methylation in vivo, once established in the ICM, may not require much maintenance or modification until gastrulation. However, the loss of PTIP in ES cells cultured in vitro may result in a

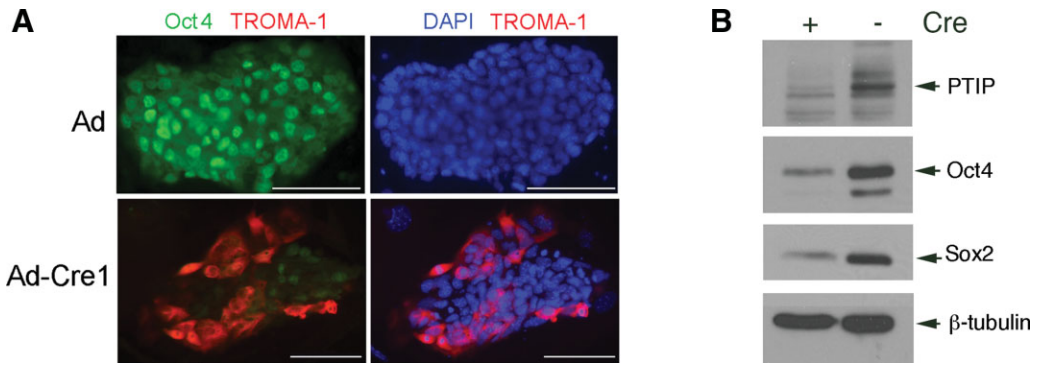

Figure 6. Protein analysis in PTIP mutants. (**A**): Immunocytochemistry of PTIP-deficient ES cells. ES cell colonies were stained with anti-Oct4 (green) and anti-TROMA-1 (red) as indicated. Right panels were counterstained with DAPI. (**B**): Protein expression analysis in mixed ES cell populations. Parental *ptip*$^{fl/fl}$ ES cells were infected with Ad-Cre or Ad alone and cultured for 96 hours in rLIF on gelatinized plates. Total protein lysates were assayed by Western blotting for the proteins indicated. Abbreviations: Ad, adenovirus; Ad-Cre, Cre-expressing adenovirus; DAPI, 4′,6-diamidino-2-phenylindole; ES, embryonic stem; PTIP, Pax transactivation domain-interacting protein; rLIF, recombinant leukemia inhibitory factor.

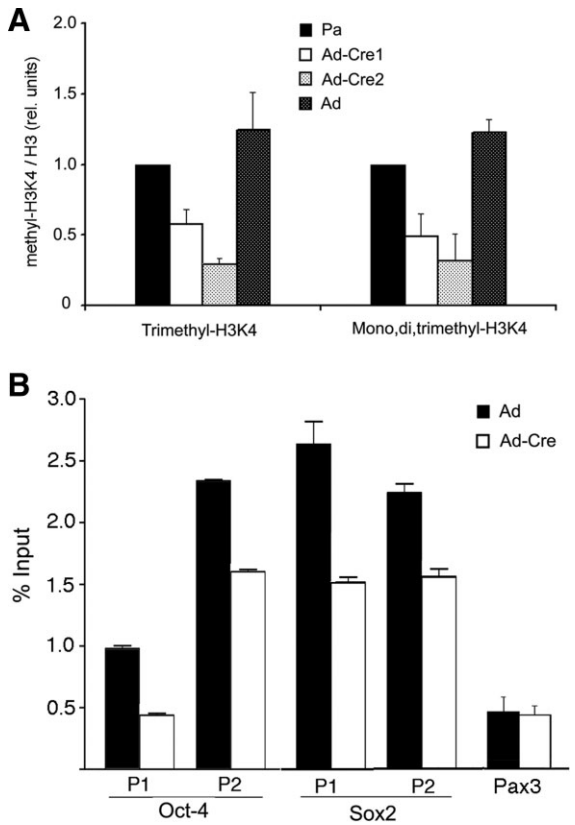

Figure 7. Analysis of histone H3K4 methylation. (**A**): Quantitative Western blotting of acid-extracted histone proteins from control and PTIP-deficient ES cells. Parental ES *ptip*$^{fl/-}$ cell lines (Pa) or *ptip*$^{fl/-}$ ES cells infected with control Ad were compared with *ptip*$^{fl/-}$ ES cells infected with Ad-Cre (Ad-Cre1, Ad-Cre2). (**B**): ChIP assay from total *ptip*$^{fl/fl}$ ES cells infected with control Ad or Ad-Cre harvested 4 days postinfection. Antibodies against trimethyl-H3K4 or control rabbit IgG were used to immunoprecipitate chromatin. ChIPed DNAs were analyzed with primer pairs for the promoter regions of *oct4*, *sox2*, and *Pax3*. The level of H3K4 methylation as a percent input was normalized to control IgG. Error bars represent one standard deviation from the mean. $p < .02$ for Ad and Ad-Cre cells using the *oct4* and *sox2* primer pairs. Abbreviations: Ad, adenovirus; Ad-Cre, Cre-expressing adenovirus; ChIP, chromatin immunoprecipitation; ES, embryonic stem; PTIP, Pax transactivation domain-interacting protein.

gradual decrease in H3K4 methylation marks as a result of the greater number of cell divisions incurred.

Whereas the necessity of Oct4 expression for maintaining pluripotency in ES cells or establishing pluriotency in induced pluripotent stem (iPS) cells is clear, the regulation of *oct4* is less well understood. Evidence for regulation by the spalt homologue Sall4 includes direct binding to a distal *oct4* enhancer and transactivation [34]. More recently, the orphan nuclear receptor Esrrb was also shown to bind to *oct4* promoter sequences to maintain expression in ES cell lines [35] and can function to reprogram fibroblasts into iPS cells when expressed with Oct4 and Sox2 [36]. However, it is not clear whether loss of these factors is ultimately responsible for *oct4* downregulation upon differentiation.

Our previous studies with PTIP indicate that it is necessary for linking the DNA-binding protein Pax2 to the MLL2/3 histone H3K4 methyltransferase complex [20]. In *Xenopus* animal caps, the PTIP homologue Swift was shown to bind to P-Smad to direct mesoderm development [37]. It is unlikely that PTIP regulates *oct4* through a Pax protein, as *pax* genes are not expressed at high levels in ES cells. Indeed, we were unable to immunoprecipitate PTIP directly to the *oct4* promoter sequences that show lower levels of H3K4 methylation. This may reflect a lack of sensitivity of our anti-PTIP antibodies. Alternatively, the reduction in H3K4 methylation could reflect lower transcription levels from the *oct4* locus resulting from the loss of an activator protein that is directly under the control of PTIP. However, given the global reduction in H3K4 methylation observed in PTIP^{-} ES cells and in the germline- and neural stem cell–specific PTIP mutants described previously [20], the data strongly argue for a direct effect on the epigenetic marks of many genes by the PTIP-containing complexes.

In summary, loss of PTIP in cultured ES cells results in lower levels of H3K4 methylation, including at the *oct4* and *sox2* promoter, reduced Oct4 protein and mRNA expression, and spontaneous differentiation toward the trophectoderm lineage. These results suggest that the continued activities of H3K4 methylation complexes are needed to maintain ES cell pluripotency in vitro.

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Disclosure of Potential Conflicts of Interest

The authors indicate no potential conflicts of interest.

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