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The BRCT-Domain Containing Protein PTIP Links Pax2 to a Histone H3, Lysine 4 Methyltransferase Complex

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Abstract

Active or repressed chromatin domains are established during development by epigenetic imprinting mechanisms that modify chromatin. The MLL family of histone methyltransferases maintain active chromatin domains by histone H3, lysine 4 methylation. How MLL complexes recognize specific chromatin domains in a temporal and tissue specific manner remains unclear. We show that the DNA binding protein Pax2 promotes assembly of an H3K4 methyltransferase complex through the ubiquitously expressed nuclear factor PTIP (Pax Transcription activation domain Interacting Protein). PTIP co-purifies with ALR, MLL3, and other components of a histone methyltransferase complex. The PTIP promotes assembly of the ALR complex and H3K4 methylation at a Pax2 DNA recognition sequence. Without PTIP, Pax2 binds to its recognition sequence but does not assemble the ALR complex. Embryonic lethal *PTIP* null mutants and conditional mutants both show reduced levels of methylated H3K4. Thus, PTIP is a novel component of a histone methyltransferase complex that links DNA binding developmental regulators to epigenetic imprinting.

Keywords

PTIP; Pax2; Histone Methyltransferase; ALR; Epigenetics

Introduction

How gene expression patterns are established and maintained during embryonic development of a complex, multicellular organism depends on the actions of epigenetic regulatory genes that impact chromatin structure and enable the inheritance of active and silent genes in specific cell lineages. First described in *Drosophila*, the Polycomb and Trithorax group genes repress or activate homeotic gene expression through the modification of chromatin structure (Ringrose and Paro, 2004). Gene silencing or activation correlates with specific post-translational modifications of histones within the nucleosome of eukaryotic chromatin. The variety of modifications, including acetylation, methylation, ubiquitination, and phosphorylation, of histone tails prompted the theory of an epigenetic code that could determine the fate of specific genes in subsequent generations of proliferating cells (Berger, 2007; Hake and Allis, 2006; Jenuwein and Allis, 2001; Margueron et al., 2005; Shilatifard, 2006). Yet, it

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remains unclear how the histone modification machinery recognizes individual genes in a temporal and tissue specific manner during vertebrate development.

Several proteins of the Trithorax and Polycomb groups have histone methyltransferase (HMT) activity that correlates with gene activity or gene silencing depending on which specific residues are modified. Within the amino-terminal tails, lysine residues available for methylation include K4, K9, K27, and K36 of histone H3 and K20 of histone H4. Methylation of H3K9 by the mammalian HMT Suv39h (Rea et al., 2000) correlates with silent chromatin (Litt et al., 2001), whereas actively expressed genes are enriched in di- (me2) and trimethylated (me3) histone H3K4 (Santos-Rosa et al., 2002). In *Drosophila*, Enhancer of Zeste and Extra Sex Combs are Polycomb group proteins that form a complex capable of methylating histone H3K27 to silence expression (Czermin et al., 2002; Muller et al., 2002). The Trithorax group proteins TRX and Ash1 contain SET domains and are associated with histone H3K4 methylation (Beisel et al., 2002; Milne et al., 2002; Nakamura et al., 2002). However, epistasis experiments suggest that Trithorax group genes function not as activators but as suppressors of silencing by the Polycomb genes (Klymenko and Muller, 2004). The complexity inherent in the pattern of histone modification is further underscored by recent findings that key developmental genes have low levels of both histone H3K4 and H3K27 methylation marks in embryonic stem cells, suggesting a bivalent chromatin domain that is activated or suppressed as differentiation proceeds (Bernstein et al., 2006).

The conserved SET domain, first described in the yeast protein Set1 (Briggs et al., 2001), is the catalytic domain for methyltransferases and is found in more than 50 mammalian proteins. Among these are homologues of the *Drosophila* Trithorax, including MLL/ALL (Milne et al., 2002; Nakamura et al., 2002), ALR (Goo et al., 2003), and MLL2 (Glaser et al., 2006). How does methylation at specific residues alter chromatin structure? The modified histone tails may interact with specific proteins such as chromatin remodeling factors that recognize methylated lysine residues (Jacobs and Khorasanizadeh, 2002; Min et al., 2003; Nielsen et al., 2002). The WDR5 protein facilitates tri-methylation of H3K4 (Wysocka et al., 2005) and subsequent interaction with the PhD finger of the BPTF subunit of a nucleosome remodeling factor (Wysocka et al., 2006). Thus, histone methylation may provide docking sites for chromatin remodeling proteins to establish and maintain open or closed configurations.

Methylation of histones must be regulated in a spatial and temporal manner as specific loci are differentially marked during development. Yet, how the histone methylation complexes recognize genes in a tissue specific manner is not clear. In this report, we show that the modular BRCT-domain protein PTIP (Pax Transcription activation domain Interacting Protein) links the developmental regulator Pax2 to the H3K4 methylation machinery. Pax2 specifies a region of mesoderm fated to become kidney and urogenital epithelium and also demarcates the midbrain-hindbrain junction (Bouchard et al., 2002; Torres et al., 1995). The Pax family of DNA binding proteins determine cell fate or patterns in the post-gastrulation embryo. PTIP was discovered because of its interactions with Pax2 (Lechner et al., 2000) but also interacts with other transcription factors (Shimizu et al., 2001). PTIP is part of a histone methyltransferase complex that includes the SET domain proteins ALR and MLL3 and other homologues of the yeast Compass complex (Miller et al., 2001). The PTIP complex localizes to a Pax2 DNA binding sequence, in a Pax2 dependent manner, and recruits the ALR methyltransferase complex to the Pax2 binding site. In PTIP siRNA knockdown cells, Pax2 still binds an integrated DNA target site, but the ALR complex fails to form and no histone H3K4 methylation occurs. Furthermore, PTIP null embryos are severely disorganize from the time of gastrulation and show a clear reduction of H3K4 staining, suggesting that PTIP is required for H3K4 methylation at many loci. Similar reductions of global H3K4 levels are observed in the developing spinal cord of conditional PTIP mutants, whereas methylation at

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histone H4K20 appears unaffected. Thus, PTIP links DNA binding proteins that specify cell lineages to an H3K4 histone methyltransferase complex.

Results

Biochemical Purification of a PTIP complex

In order to better understand the function of PTIP, we purified PTIP containing nuclear complexes. HEK293 cells were transfected with flag-tagged PTIP or control vectors. Some PTIP expressing cells were subject to 10 Gy gamma irradiation because PTIP had been shown to interact with proteins in the DNA damage response pathway. Nuclear extracts were prepared from 200 plates of cells as outlined (Fig. 1A). After initial purification over P11 phosphocellulose and DEAE Sephacel columns, proteins were bound to M2 flag agarose and eluted with 3x flag peptide. Elutions were tested for PTIP using anti-flag and anti-PTIP antibodies and pooled for further analysis. Concentrated elutions were separated on 12% SDS/PAGE gels and stained with colloidal coomasie (Fig. 1B). Proteins co-purifying with PTIP were analyzed by mass spectrometry and peptide sequences determined. The identities of specific proteins and the number of peptides sequenced for each protein are listed (Table 1).

Among the high molecular weight proteins in the PTIP complex were human ALR and MLL3, with more than 65 and 57 peptide sequences respectively. ALR and MLL3 are related to MLL and contain SET domains, which are associated with Histone methyltransferase activity. Many of the proteins in the PTIP complex(s), such as Ash2L, WDR5, Rbbp5, and SMC1 are associated with Histone modification. In order to confirm these data, we co-immunoprecipitated endogenous proteins from HEK293 nuclear extracts. Chicken anti-PTIP also pulled down Ash2L, WDR5, and Rbbp5 (Fig. 1 C-E). Reciprocal immunoprecipitations with anti-Ash2L and anti-Rbbp5 also co-precipitated PTIP. Anti-WDR5 was not efficient for immunoprecipitation since it does not recognize the native form of the protein (J. Wysocka, personal communication). Antibodies against ALR were able to co-immunoprecipitate endogenous PTIP, Ash2L, and Rbbp5 (Fig. 1 F). Finally, antibodies against PTIP, Ash2L, Rbbp5, and NCOA6 were able to co-immunoprecipitate most of the proteins in the complex (Fig. 1 G). Thus, the PTIP complex includes ALR, Ash2, WDR5, Rbbp5, and NCOA6.

The PTIP Complex Methylates Histone H3

The purified PTIP fractions were able to methylate histone H3, core histones and mononucleosomes in vitro (Fig. 2A). Irradiation did not dampen the ability of the PTIP complex to methylate, if anything there is more methylation of recombinant H3 and core histones. Furthermore, we used antibodies against specific modifications of histone H3 to determine that methylation was at K4 (Fig. 2 B). Antibodies against H3K9me2, H3K27me2, H3K27me3, H4K20me1, or H4K20me2 did not recognize *in vitro* methylated substrates in these assays (data not shown). To confirm the specificity and types of modifications mediated by the PTIP complex, we developed an immunolinked histone methylation assay. Both transfected and endogenous immunoprecipitated PTIP complexes methylated histone H3 (Fig. 2 C, D). Antibodies specific for different modifications confirmed that PTIP immunoprecipitates can mono-, di-, and trimethylate H3K4 (Fig. 2 E). Nuclear lysates from E17.5 kidneys confirmed that PTIP interacts with Ash2L and Rbbp5 *in vivo* (Fig. 2 F). The PTIP from tissues methylated H3K4 using purified monocucleosomes as substrates (Fig. 2 G). When compared to recombinant Set9 as a reference, IPs from embryonic tissue had more methyltransferase activity than tissue culture cells.

PTIP is a modular protein with 3 pairs of BRCT domains and a domain rich in poly-glutamines. To determine which domain of PTIP associated with methyltransferase activity, we analyzed deletion mutants using the immunolinked methylation assay (Fig. 3). Epitope tagged forms

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containing the carboxyl terminal BRCT domains (5 and 6) were sufficient to immunoprecipitate the H3K4 methyltransferase activity, whereas deletion of these two BRCT domains generated a protein unable to co-precipitate the activity (Fig. 3 C).

Formation of the PTIP methyltransferase Complex at a Pax2 Response Element

Since the PTIP protein interacts with the carboxy-terminal domain of Pax2 (Lechner et al., 2000), we examined the localization of PTIP and other proteins of the methylase complex at a Pax2 binding element in cells. Since there are few known Pax2 target genes that respond well in transiently transfected cells, we designed an EGFP reporter system in HEK293 cells that contained five Pax2 binding elements, upstream of a minimal promoter (Fig. 4 A). This reporter was stably integrated into cells and individual clones screened for Pax2 responsiveness. Multiple clonal lines were established that activated EGFP expression in response to transient Pax2 transfection (Fig. 4 B). The clones showed a dose response, as increasing amounts of Pax2 activated the integrated EGFP reporter construct (Fig. 4 C).

Chromatin from control or Pax2 transfected cells was prepared 48 hours post transfection for immunoprecipitation assays (CHIPs). Isolated chromatin was immunoprecipitated using a variety of different antibodies, as indicated (Fig. 4 D). PCR primer pairs against either the PRS4 Pax2 binding region or the promoter of the GAPDH gene were used to determine the degree of enrichment relative to input chromatin. The CHIPs data indicate that Pax2 binds to the PRS4 sequence, as expected. Furthermore, PTIP and the other proteins of the methylase complex, including Ash2L, ALR, and Rbbp5, localize to the PRS4 sequence in a Pax2 dependent manner. This results in a more than 10 fold increase in H3K4 mono- and dimethylation and a five fold increase in histone H3K4 trimethylation at the Pax2 binding element (Fig. 4 D). No significant differences were observed at the GAPDH promoter. These results show PTIP can localize to a Pax2 binding element in a Pax2 dependent manner. The increase in histone H3K4 methylation is also Pax2 dependent and likely to be the result of the PTIP methylase complex forming at the binding site.

PTIP is Essential for Linking Pax2 to the ALR Complex

We next asked whether PTIP was necessary for the formation of the H3K4 methyltransferase complex at the PRS4 Pax2 binding element. Using siRNAs, PTIP was knocked down in the PRS4-EGFP cell lines that were also transfected with Pax2 (Fig. 5 A). The PTIP knockdowns could be rescued by expression of a mouse PTIP that was resistant to the human siRNAs. After 40 h, GFP expression was up in Pax2 transfected cells, but remained at base line in PTIP knockdown cells. However, mouse PTIP could rescue the Pax2 mediated activation of GFP expression.

The assembly of the PTIP/ALR complex was assayed by chromatin immunoprecipitation (Fig. 5 B-J). Pax2 binding to the PRS4 sequence was unaffected in PTIP knockdowns. However, none of the proteins tested in the ALR H3K4 complex, including ALR, Ash2L, or Rbbp5, could localize to the PRS4 sequence in the absence of PTIP. Consistent with this observation, PTIP knockdowns did not show Pax2 dependent increase in H3K4 methylation at the PRS4 sequence. Transfected mouse PTIP could restore the assembly of the ALR complex at PRS4 and restore H3K4 methylation, even in the presence of human PTIP siRNAs. These data demonstrate that PTIP is required for the assembly of an ALR H3K4 methyltransferase complex at a Pax2 binding site within chromatin.

PTIP Null Embryos or Tissues Exhibit Altered Histone H3 Methylation Levels

In the mouse, PTIP null embryos are disorganized and developmentally arrested from the time of gastrulation (Cho et al., 2003). The phenotype encompasses more than the Pax2 expression domain, suggesting that PTIP interacts with additional factors. Despite the delayed

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development, some tissues are still evident at E9 including neural ectoderm, cardiac tissue and the occasional somite, though the degree of development varies. Given the function of PTIP in H3K4 methylation, we stained embryo sections with antibodies against H3K4me2,3 to determine if the global levels of these modifications were affected (Fig. 6). Sections through the developing neural tube, somites and limb buds of an E9 wild-type embryo showed strong nuclear staining with anti-H3K4me2 and anti-H3K4me3 in most all cells (Fig. 6B, C). In contrast, PTIP$^{-/-}$ embryos showed reduced staining with both antibodies (Fig. 6 G, H). This was most evident in the presumptive neural tissue of the head folds and the mesodermal derivatives. The extraembryonic tissues appeared unaffected, as strong nuclear staining, comparable to wild-type, was observed in the trophoblast nuclei of PTIP$^{-/-}$ embryos (Fig. 6 K, L). A few cells within the neural ectoderm did show normal levels of staining (Fig. 6 G, H, red arrows), but these were rare. Despite the disorganized nature and developmental delay of the PTIP null embryo, nuclear staining with DAPI indicated many cells were intact and exhibited large, round nuclei with distinctly visible nucleoli. The data suggest that PTIP is essential for the initiation or maintenance of normal levels of histone H3K4 methylation marks at this stage of development.

The *PTIP* null allele is early embryonic lethal and causes significant disorganization, arrest, and cell death. Thus, we also generated a *PTIP* conditional allele to assess the effects of *PTIP* deletion at later developmental stages (Fig. 7). Homozygous *PTIP$^{loxP/loxP}$* mice were viable and fertile, with no distinguishable phenotype. We deleted PTIP in the neuroepithelium of the developing spinal cord using a Nestin-Cre driver strain active at E10 (Sclafani et al., 2006;Trumpp et al., 1999). By E17.5, the Nestin-Cre:*PTIP$^{loxP/loxP}$* mice had reduced levels of PTIP in the spinal cord (Fig. 7 B, lanes 2 and 6), whereas the liver was unaffected. Of 71 newborns genotyped, none of the 14 Nestin-Cre:*PTIP$^{loxP/loxP}$* animals survived more than one day. The exact cause of post-natal lethality has not been determined. However, we have examined the levels of histone methylation in the spinal cord and in other tissues in order to confirm what was observed in at E9.0 in the PTIP$^{-/-}$ embryos.

In *PTIP$^{loxP/loxP}$* embryos at E17.5, strong nuclear staining is observed in the spinal cord and most all developing tissues using antibodies against H3K4me2 or H3K4me3 (Fig. 7 C). However, in *PTIP$^{loxP/loxP}$* animals that also carry the Nestin-Cre transgene, this H3K4me2,3 staining was clearly reduced (Fig. 7 D). This reduction was observed in the spinal cord but not in other tissues, such as skin, hair follicles and the developing tongue of the mandibular region (Fig. 7 C). Levels of methyl Histone H4K20me1 were not significantly changed when comparing *PTIP$^{loxP/loxP}$* embryos to Nestin-Cre:*PTIP$^{loxP/loxP}$* embryos. These results indicate that loss of PTIP reduces the global levels of histone H3K4 methylation in developing tissues and suggest an essential role for PTIP in the methylation of a large number of loci.

Discussion

A ubiquitously expressed, nuclear protein essential for embryonic development, PTIP is part of a complex containing the histone methyltransferases ALR and MLL3 and proteins of the mammalian COMPASS-like complex, Ash2L, WDR5, Rbbp5, and NCOA6 (Miller et al., 2001; Steward et al., 2006). The purified PTIP complex can methylate histone H3K4 *in vitro*. Recently, a similar PTIP histone methyltransferase complex was described by Cho et al (Cho et al., 2007) who also identified a novel component, PA1. Characterization of the ALR complex by Issaeva et al. (2007) also identified PTIP as a co-factor. However, the function of PTIP within the complex was not directly addressed in either study. Our data indicate that PTIP is an essential component of the H3K4 methyltransferase complex and mediates assembly of the complex at specific DNA sequences. The data link epigenetic modifications at specific histone residues to DNA binding proteins that specify cell lineages during development.

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PTIP, Gastrulation, and Epigenetics

In mammals, gastrulation converts the pluripotent epiblast into the three primary germ layers, endoderm, ectoderm, and mesoderm. Prior to this point, differentiation and cell lineage restriction reflects the specification of the extraembryonic tissues, such as the trophoblasts and extra-embryonic ectoderm. The phenotype of *PTIP* null embryos is restricted to the epiblast derived structures, as extraembryonic tissues show normal levels of histone H3K4 methylation. Similar observations were made previously, as TUNEL positive cells were only evident within the embryo proper at E7.5−8.5 (Cho et al., 2003). Other genes in epigenetic pathways also exhibit abnormal gastrulation and germ layer specification. Loss of either murine Polycomb homologues *eed* (Faust et al., 1998) or *Ezh2* (O'Carroll et al., 2001) affect gastrulation and mesoderm differentiation. The histone H3K9 methyltransferase ESET is required even earlier for blastocyst formation and implantation (Dodge et al., 2004). Three different mutant alleles of the histone H3K4 methyltransferase *Mll*, which regulates Hox gene expression (Milne et al., 2002), have been generated. Hox gene expression changes, altered patterns of Hox promoter histone methylation, and homeotic transformations are observed in two mutants (Terranova et al., 2006; Yu et al., 1995), whereas a third is early preimplantation lethal (Ayton et al., 2001).

Development of *Mll2*$^{-/-}$ mice arrests post gastrulation at E7.5 (Glaser et al., 2006). Altered Hox gene regulation was observed but unlike *Mll*, which regulates *Hoxc8*, *Mll2* regulates expression of the *Hoxb2* and *Hoxb5* genes. While mouse MLL2 is the homologue of human MLL4, the *Mll2*$^{-/-}$ phenotype resembles, in part, the *PTIP* null allele, especially the timing and stochastic nature of cell loss. The genetics point to an essential role for the mouse Mlls and PTIP in maintaining early patterning during and post gastrulation, whereby the Set domain proteins Mll, ALR, Mll2, and Mll3 are likely to have different target gene specificity.

In the developing spinal cord, the ventricular neuroepithelial cells generate all of the specialized neurons and glia of the central nervous system. While they are lineage restricted, they represent a population of neuronal stem cells that can renew and differentiate. Loss of PTIP in these stem cells also results in reduced levels of histone H3K4 methylation. The effects of PTIP deletion are complex, as the cause of the observed postnatal lethality has not been definitively determined. Of note, we have generated several other PTIP tissue specific deletions at different developmental stages. Deletion of PTIP in epithelial cells of the renal medulla result in urine concentration defects, consistent with a failure to respond to hormonal inputs (Kim et al., 2007). Adult animals that have deleted PTIP in the liver, using Albumin-Cre, and in the podocytes of the kidney, using Nephrin-Cre, are both viable and fertile (Kim and Dressler, unpublished). PTIP deletion in these strains occurs late in development, in more terminally differentiated cells, suggesting that once epigenetic patterns are established PTIP may no longer be required.

PTIP and DNA Binding Proteins

For histone methylation complexes, the ability to recognize and alter chromatin in a locus and tissue specific manner must require interactions with sequence specific DNA binding proteins. During development, genes that encode DNA binding proteins specify cell lineages or position along the body axes. Such genes include the homeotic or Hox genes, the Pax genes, the helix-loop-helix and zinc finger families, the forkhead genes, and many others. Fate mapping and transplantation studies in chick and mouse embryos suggest that initial developmental decisions are made during gastrulation as cells move through the primitive streak (Le Douarin et al., 1997; Lemaire and Kessel, 1997; Robb and Tam, 2004). The decision to become mesoderm or neural ectoderm is generally thought to be irreversible and must rely on epigenetics to maintain cellular memory within a rapidly proliferating population. Epigenetic modifications must be regulated by DNA binding proteins that provide locus and tissue specificity. Given its modular structure, we propose that PTIP provides a link between some

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developmental regulatory proteins that specify cell lineages or patterns and the H3K4 histone modification machinery.

The current state of chromatin immunoprecipitation technology does not readily allow for the analysis of histone modifications at individual loci in small amounts of embryonic tissues. The use of cell lines can circumvent this problem, but has limitations. Introduction of a developmental regulatory protein such as Pax2 into HEK293 cells has little effect on the endogenous gene expression patterns. The most likely explanation is that established cell lines lack competence to respond, due to inherent epigenetic repressive marks already accumulated. The idea of competence to respond to inductive signals is a long established concept in development and can be modeled along epigenetic principles. This is illustrated by recent findings in ES cells, which are competent to respond to many inductive inputs, where neither high levels of negative or positive epigenetic marks were observed on critical developmental regulatory genes until a cell fate decision was made (Bernstein et al., 2006; O'Neill et al., 2006). To overcome the lack of competence in HEK293 cells, we engineered and selected for a Pax2 responsive gene that was epigenetically naive. This allowed us to characterize the biochemistry of H3K4 methyltransferase assembly in a model Pax2 dependent system. However given the developmental defects and reduction of H3K4 methylation observed in PTIP mutants, it is likely that PTIP interacts with many other DNA binding proteins.

Regulation of PTIP Interactions

PTIP contains 3 pairs of BRCT domains, at least some of which are Phospho-Serine (P-S) binding (Manke et al., 2003; Clapperton et al., 2004; Shiozaki et al., 2004; Yu et al., 2003). The Pax2 protein contains a serine-threonine rich carboxy terminus that can be phosphorylated by WNT signals and the c-Jun N-terminal kinase (Cai et al., 2003; Cai et al., 2002). During kidney development, a process entirely dependent on Pax2 function (Torres et al., 1995), WNTs are the inducing signals that promote aggregation and polarization of the intermediate mesoderm into kidney epithelial cells (Carroll et al., 2005; Stark et al., 1994). In *Xenopus* development, the PTIP homologue interacts with Smad2 in a TGF-β dependent manner, suggesting that phosphorylation of Smad2 is necessary for this interaction (Shimizu et al., 2001). These examples suggest that positional information, as specified by extrinsic signaling molecules, can be translated epigenetically by phosphorylation of DNA binding proteins that promote histone methylation at specific loci.

Several prior studies also suggest that PTIP functions in the DNA damage response pathways and can interact with 53BP1 at nuclear foci after ionizing radiation (Jowsey et al., 2004; Manke et al., 2003). These interaction may be mediated by the carboxyl-terminal BRCT domains which preferentially bind to P-SQ, a substrate for the ATM/ATR kinases (Manke et al., 2003). Consistent with these data, we co-purified both 53BP1 and Rad50 with PTIP in cells exposed to ionizing radiation. Cho et al (2007) show that PTIP associates with two independent complexes, an ALR/Mll3 containing histone methyltransferase complex and a DNA damage response complex. Previous experiments with more terminally differentiated trophoblast cells also demonstrated increased sensitivity to ionizing radiation upon loss of PTIP (Cho et al., 2003). While these data suggest a role for PTIP in the DNA damage response, the significance of the interaction with 53BP1 and localization to nuclear foci remain unclear.

Pax Proteins, Cell Lineages, and Epigenetics

That Pax proteins promote the accumulation of active epigenetic marks has not been reported, although the data are consistent with the known biological functions in cell lineage determination and cell fate restriction during development. For example, the conserved *Pax6* gene specifies the region fated to become the eye in both vertebrates and flies (Chow et al., 1999; Quiring et al., 1994), whereas the *Pax2* gene can specify the intermediate mesoderm that

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will generate the urogenital tract (Bouchard et al., 2002; Torres et al., 1995). Cell lineage restriction must involve the accumulation of both positive and negative epigenetic marks, among which histone H3K4 methylation is just one example.

In summary, we provide biochemical and genetic evidence that PTIP is an essential component of an ALR histone H3K4 methyltransferase complex. PTIP is necessary for assembly of the ALR complex and methylation of H3K4 in response to binding of Pax2 to its DNA recognition sequence within the context of chromatin. These data suggest that developmental regulatory proteins, such as Pax2, provide the locus and tissue specificity for epigenetic modifications during development.

Experimental Procedures

Plasmids

Full length pMYC-PTIP has been described (Lechner et al., 2000). The plasmid p3XFLAG-PTIP was made by inserting a *Not1* fragment of pMYC-PTIP into the *Not1* site of 3X FLAG CMV 10 (E4401, Sigma). pMYC-PTIP SH, which encodes amino acids 5 to 591, was made by inserting a *SmaI-HindIII* fragment from pPC86-PTIP (Lechner et al, 2000) into *SmaI* and *HindIII* sites in the mammalian expression vector pMYCrk5. pMYC-PTIP SX, encoding amino acids 5 to 796, was created by inserting a *SmaI-XbaI* fragment from pPC86-PTIP into *SmaI* and *Xba1* sites in pMYCrk5. pMYC-PTIP XS, encoding amino acids 796 to 1056, was created by inserting an *XbaI-SpeI* fragment from pPC86-PTIP into an *XbaI* site in pMYCrk5. FLAG-PTIP BRCT 5,6 was created by inserting an *XbaI-EcoRI* fragment of from pPC86-PTIP into *XbaI-EcoRI* sites of3X FLAG CMV 10. Recombinant Set 9 was a kind gift of R. Trievel.

Antibodies

The chicken anti-PTIP antibodies have been described (Lechner et al., 2000). Rabbit anti-PTIP antibody was generated against GST-fused mouse PTIP residues 316−591 and purified on protein A agarose (Harlow and Lane, 1988). Anti-chicken IgY (N-1010) and chicken IgY agarose (Preciphen, P-1010) were purchased from Aves labs. Anti-c-MYC 9E10 (MMS-150R) and anti-HA (MMS-101P) were from Covance. Rabbit IgG (I-5006), goat IgG (I-5256), anti-FLAG (F-3165) and anti-M2 FLAG agarose (A2220) were from Sigma. Anti-Ash2L (A300 −107A), anti NcoA6 (A300−410), anti-Rbbp5 (A300−109A) were from Bethyl Labs. Goat anti-human ALR/Mll2 (ab15962), anti-H3K4me1/2/3 (ab8895, ab7766 and ab8580) were purchased from Abcam. Rabbit anti-WDR5 was a gift of J. Wysocka and C.D. Allis.

Cell Culture, Transfection and Cell Lysate Preparation

HEK 293 cells (ATCC) were cultured in Dulbecco's modified Eagle's medium (glucose concentration, 450 mg/ dl) supplemented with 10% fetal bovine serum and 100 U/ml penicillin and 100 mg/ml streptomycin in 5% CO2/95% air at 37°C. Cells in 10 cm plates were transfected using 1−3 μg of total plasmid DNA and 12 μl of Fugene 6 as described (Roche Molecular Biochemicals). Forty hours after transfection, cells were harvested and either nuclear extracts or whole cell lysates were prepared (Patel and Dressler, 2004). Whole cell lysates for immunoprecipitation and immuno-linked methyltransferase assays were prepared in ice-cold buffer containing 50 mM Tris pH 8.0, 0.4 M NaCl, 1 mM ETDA, 0.5% NP40 with a cocktail of protease inhibitors (Roche Molecular Biochemicals). The cells were rotated at 4 °C for 30 minutes and then centrifuged for 20 min at 13,000 rpm at 4 °C to remove debris. The protein concentration was adjusted to approximately 2 mg/ml in IP buffer in 50 mM Tris, pH 8.0, 150 mM NaCl, 1 mM EDTA, 0.1% NP40 and protease inhibitors.

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HEK293 cells were co-transfected with PRS4-EGFP and a neomycin expression plasmid and selected with 400 ug/ml G418. Clones were picked, expanded and screened for EGFP expression in response to Pax2 transient transfection using a fluorescent microscope.

Protein Purification and Mass Spectrometry

200 × 10 cm plates of HEK 293 cells were transfected with p3XFLAG-PTIP or controls. An additional 200 plates of cells transfected with p3XFLAG-PTIP were subjected to 10 Gy gamma-irradiation at a rate of 200 rads/min using a Cesium^{137} gamma-irradiation source 39 hours after transfection. The cells were allowed to recover for one hour at 37°C prior to harvesting. Nuclear extracts prepared as previously described were dialyzed overnight into Buffer A (20 mM Tris-HCl, pH 7.9, 0.2 mM EDTA, 10 mM βME, 10% glycerol, 0.2 mM PMSF, 0.1M KCl). Approximately 40 mg of each nuclear extract was loaded on a 10 ml column of phosphocellulose (P11, Whatman) and fractionated stepwise in buffer A that had increasing concentrations of KCl (0.1 M wash, 0.3 M, 0.5 M, 0.7 M and 0.9 M elutions) as described (Bochar et al., 2000). The P11 0.3 and 0.5 M KCl fractions, which contained the peak PTIP proteins, were loaded on a 6 ml DEAE-Sephacel column (Pharmacia) and similarly eluted with in a stepwise fashion with increasing concentrations of KCl in buffer A. The fractions with the peak amounts of PTIP were pooled and dialyzed against buffer BC (20 mM HEPES, pH 7.9, 0.2 mM EDTA, 0.5 mM DTT, 20% glycerol, and 0.2% NP-40) with 100 mM KCl, as described (Zhang et al., 2002), and incubated overnight with anti-M2 FLAG agarose at 4 °C. The beads were washed twice with 5 column volumes of BC with 300 mM KCl. The beads were further washed four times with five column volumes of BC with 100 mM KCl. Bound proteins were specifically eluted at 4 °C with 3X-FLAG peptide (SIGMA) at a concentration of 150μg/ml. The eluted proteins were concentrated on Microcon columns (Millipore) and resolved on a 12% SDS-PAGE gel. The gel was stained with Colloidal Commassie stain (Invitrogen) and unique bands were excised and sequence analysis was performed at the Harvard Microchemistry Facility by microcapillary reverse-phase HPLC nano-electrospray tandem mass spectrometry on a Finnigan LCQ DECA XP Plus quadrupole ion trap mass spectrometer.

Co-immunoprecipitation and Immunoblotting

For immunoprecipitation, 2 mg of HEK 293 whole cell lysate (WCL), was cleared with PrecipHen (for chicken antibodies), IgA agarose (for rabbit and mouse antibodies) or sepharose G (Amersham Biosciences; for goat antibodies). 2 to 4 μg of antibody was incubated with the lysates overnight and protein-complexes were immobilized on agarose beads. After extensive washing with IP buffer, the beads were boiled in 2X Lamelli sample buffer and separated by SDS-PAGE with 1 to 2 % input. Immunoblotting was performed as previously described (Patel and Dressler, 2004).

Histone Methyltransferase (HMT) Assay

^{3}H-HMT assay was performed exactly as described (Hughes et al., 2004) with 1 ug recombinant histone H3 (Upstate), 4 ug core histones (Upstate) or 4 ug mononucleosomes (gift of D. Bochar) as substrates. Positive control reactions contained 1 ng of recombinant Set9.

Immunolinked HMT assay was performed similarly. In brief, protein-antibody complexes bound to beads were equilibrated in HMT assay buffer, and incubated with 2 ug of Histone H3 with unlabelled S-adenosyl-L-methionine (Sigma) at a final concentration of 1μM. After a 90 minute incubation at 30°C, the reaction was terminated by boiling the beads with 6X SDS sample buffer. The samples were separated on 15%

SDS-PAGE gels, and immunoblotting was performed with specific antibodies as described.

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RNA interference and rescue of PTIP in HEK 293 cells

The hPTIP siRNA sequences described by Jowsey et. al. (2004) were purchased from Dharmacon. HEK 293 with an integrated PRS4EGFP reporter were transfected with SI20 (UGC ACU AGC CUC ACA CAU AdTdT) or a scrambled version of SI20 (SCR) (CCU ACU AAG CGA CAC CAU UdTdT) siRNAs at a final concentration of 200 nM using calcium-phosphate precipitation. After 24 hours, cells were split 1:4 and retransfected with siRNA. Maximal depletion was 48 to 60 hours after the second transfection. 24 hours after the second siRNA transfection, cells were transfected with 1 μg mouse CMV-Pax2 as described (Cai et al, 2002). PTIP expression was rescued in the knockdown cells by co-transfecting 2 μg of 3XFLAG-PTIP along with CMV-Pax2. 48 hours after transfection with plasmids, cells were harvested for protein expression and ChIP experiments as described below.

Chromatin Immunoprecipitation and Real-time PCR

HEK 293 cells with an integrated PRS4EGFP reporter were transfected with 1 ug CMV-Pax2 (Cai et al, 2002) and harvested 48 hour after transfection. A fraction of the cells were lysed in 2X-SDS PAGE buffer to determine expression of Pax-2, tubulin and GFP. ChIP was performed in triplicate according to published protocols from Upstate Biotech with minor modifications. Cells were fixed for 10 min at 25°C with 1% formaldehyde in culture medium. Cross-linking 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, and protease inhibitors), incubated at 4°C for 5 minutes and centrifuged for 5 minutes at 3000 X 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 s pulses using a microtip probe sonicator (Branson Sonifier 250) with output control set to 1. Sonicated lysates were clarified by centrifugation at 4°C fro 15 minutes. 20 μg 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 and 167 mM NaCl) and preclarified with 80 ul protein A-agarose or protein G-agarose (Upstate). Each immunoprecipitation was performed with 2−5 μg antibodies. After overnight incubation at 4° C, 60 μl protein A-agarose or protein G-agarose were added and the incubation was continued for 1 h. The beads were sequentially washed two times in IP dilution buffer, two times in TSE-500 wash buffer (0.1% SDS, 1% Trition X-100, 2nM 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% Na deoxycholate), and finally two times in Tris-EDTA. Bound complexes were eluted by vortexing beads twice for 15 min at 25°C in 250 μl of elution buffer (50 mM Na bicarbonate and 1% SDS). 5M NaCl was added to a final concentration of 0.2M to the pooled eluates and cross-links reversed by incubating samples at 65°C overnight. The samples were digested with proteinase K for 1 hr 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). The sequences of the PRS4 primer pair are 5' GCTACCGGACTCAGATCTCG 3' and 5' TGCGAAGTGGACCTCGGACC 3', and GAPDH 5' TGCGTGCCCAGTTGAACCAG 3' and 5' AACAGGAGGAGCAGAGAGCGAAGC 3'. The data are represented as Mean +/− SEM of two independent experiments.

Immunostaining

Generation of the *PTIP* null allele has been described (Cho et al., 2003). Embryos from timed matings were collected and fixed in 4% paraformaldehyde and embedded in paraffin. Sections were cut at 5 microns, dewaxed and rehydrated. Antigens were unmasked with VectaLabs Antigen retrival system by boiling for 8 minutes. Primary anti-H3K4me2 and anti-H3K4me3 (AbCam) were incubated for 2 hours in PBS, 0.1% Tween-20, 2% goat serum followed by two

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washes in PBS, 0.1% Tween-20. Goat anti-rabbit FITC conjugates (Sigma) were incubated for 1 hour and washed. Micrographs were taken with a Nikon ES800 fluorescent microscope and digital Spot camera. All exposure times were set manually and were equivalent among sections.

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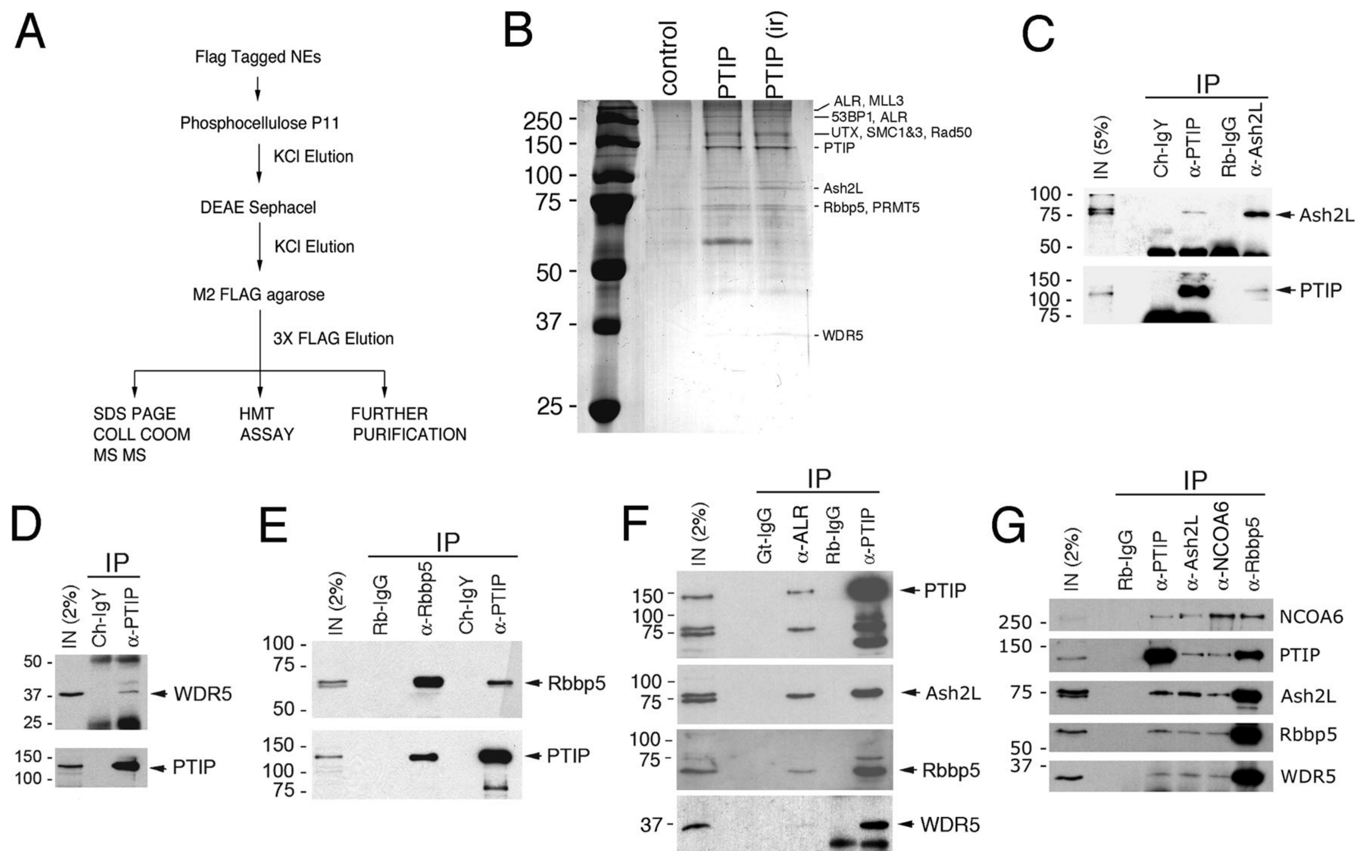

Figure 1. PTIP Co-purifies with a Histone Methylatransferase Complex

A) The biochemical purification of PTIP and associated proteins is shown. B) The final 3x FLAG elutions were concentrated, run on a 12% SDS/PAGE gel, and stained with colloidal coomasie blue. The proteins identified by MS/MS are indicated. The abundant protein at 60 kD in the PTIP lane was a Keratin contaminant. C) Chicken antibodies against PTIP or rabbit anti-Ash2L were used to immunoprecipitate (IP) from nuclear extracts directly. Co-precipitated proteins were detected by western blotting as indicated. Controls antibodies for the IP are chicken (Ch) IgY and rabbit (Rb) IgG. D) WDR5 co-immunoprecipitates with anti-PTIP but not with control antibodies, as indicated. E) Rbbp5 co-immunoprecipitates with anti-PTIP antibodies. Similarly, PTIP is co-immunoprecipitated with antibodies against Rbbp5. F) Antibodies against human ALR were used to co-immunoprecipitate PTIP, Ash2L, and Rbbp5. Similarly, rabbit anti-PTIP co-precipitates Ash2L, Rbbp5, and WDR5. Very weak signals are observed with anti-WDR5 in the ALR IPs. F) Elements of the Methyltransferase complex co-precipitate with each of the components. Antibodies against PTIP, Ash2L, NCOA6, and Rbbp5 were used to IP from whole cell lysates and western blotted with the indicated antibodies. Note, all elements of the complex are present regardless of which antibody is used, whereas control rabbit IgGs do not precipitate any proteins of the complex.

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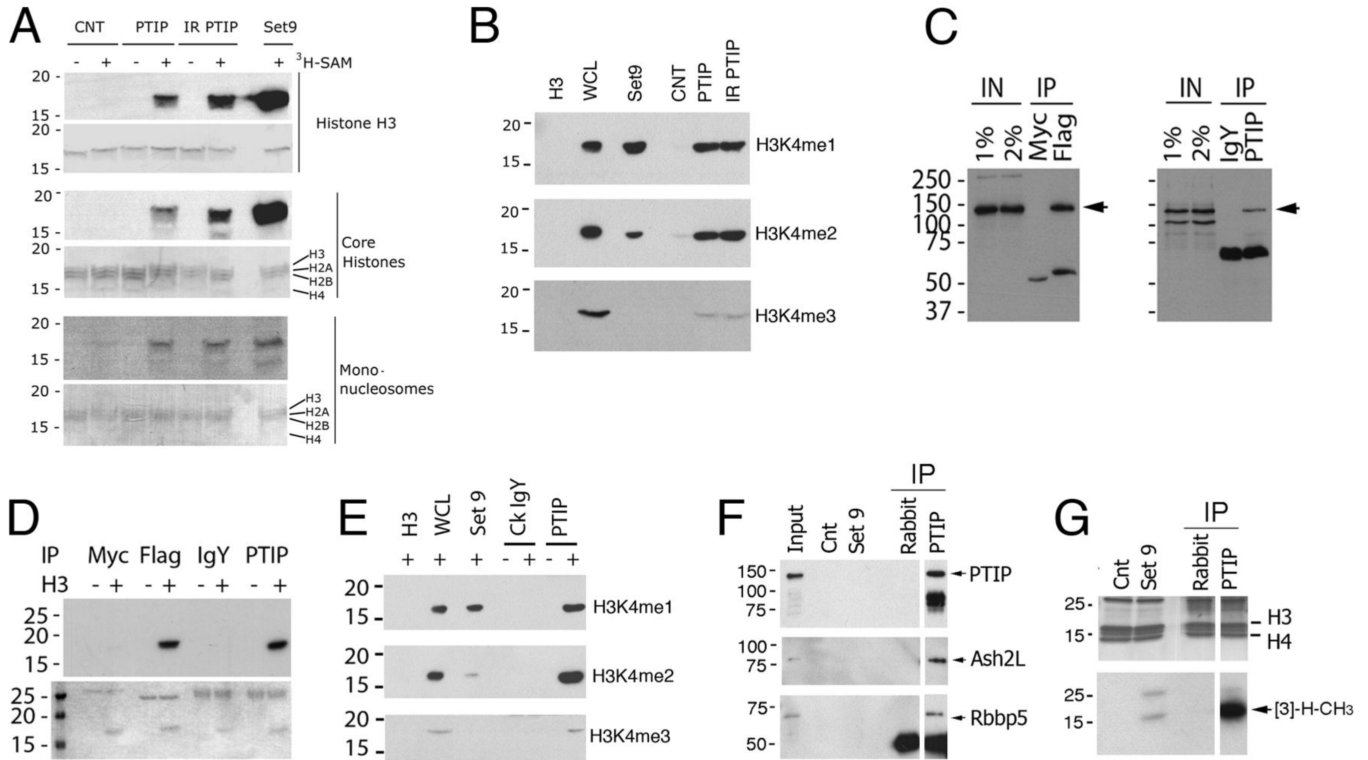

Figure 2. PTIP Associated Methyltransferase Activity

A) Methyltransferase assay using final 3X FLAG elutions isolated from PTIP transfected cells, ^{3}H-SAM, and either Histone H3, core histones, or mononucleosomes as substrates. Flourograms are shown on top and coomasie stained gels below. Control elutions (CNT) and recombinant Set9 proteins were used as negative and positive controls. B) Western blots of HMT assays using cold SAM and histone H3 as a substrate. Blots were probed with antibodies specific for mono, di-, or trimethyl H3K4 as indicated. Control lanes include unmodified histone H3 (H3), whole lysates (WCL) or recombinant Set9 (Set9). Purified PTIP from expressing (PTIP) and irradiated cells (IR-PTIP) is able to methylate H3K4, whereas control (CNT) elutions do not. C) Immunoprecipitation of Flag-PTIP or endogenous PTIP is indicated (arrow). These PTIP containing beads were used for immunolinked methylation assays in D. D) Methylation assays using ^{3}H-SAM and Histone H3 (+) as a substrate and immunoprecipitated PTIP. Fluorogram is shown on the top panel and coomasie stained gel is below. Note methylated Histone H3 with anti-Flag and anti-PTIP antibodies but not with controls (anti-myc or IgY). E) Similar assay as in D but using unlabeled SAM. Methylation reactions were run on 15% SDS/PAGE gels and western blotted with antibodies specific for mono-, di-, and trimethyl K4 of Histone H3 as indicated. F) Nuclear lysates were prepared from E17.5 embryonic kidneys and immunoprecipitated (IP) with anti-PTIP and control antibodies. Co-precipitating proteins were analyzed by western blotting with the antibodies indicated. Note, PTIP, Ash2L, and Rbbp5 co-precipitate from embryonic kidney nuclear extracts. G) The PTIP IPs from embryonic kidney (panel F) were also used to methylate purified mononucleosomes with ^{3}H-SAM, coomasie stained gel is on top and fluorogram is below.

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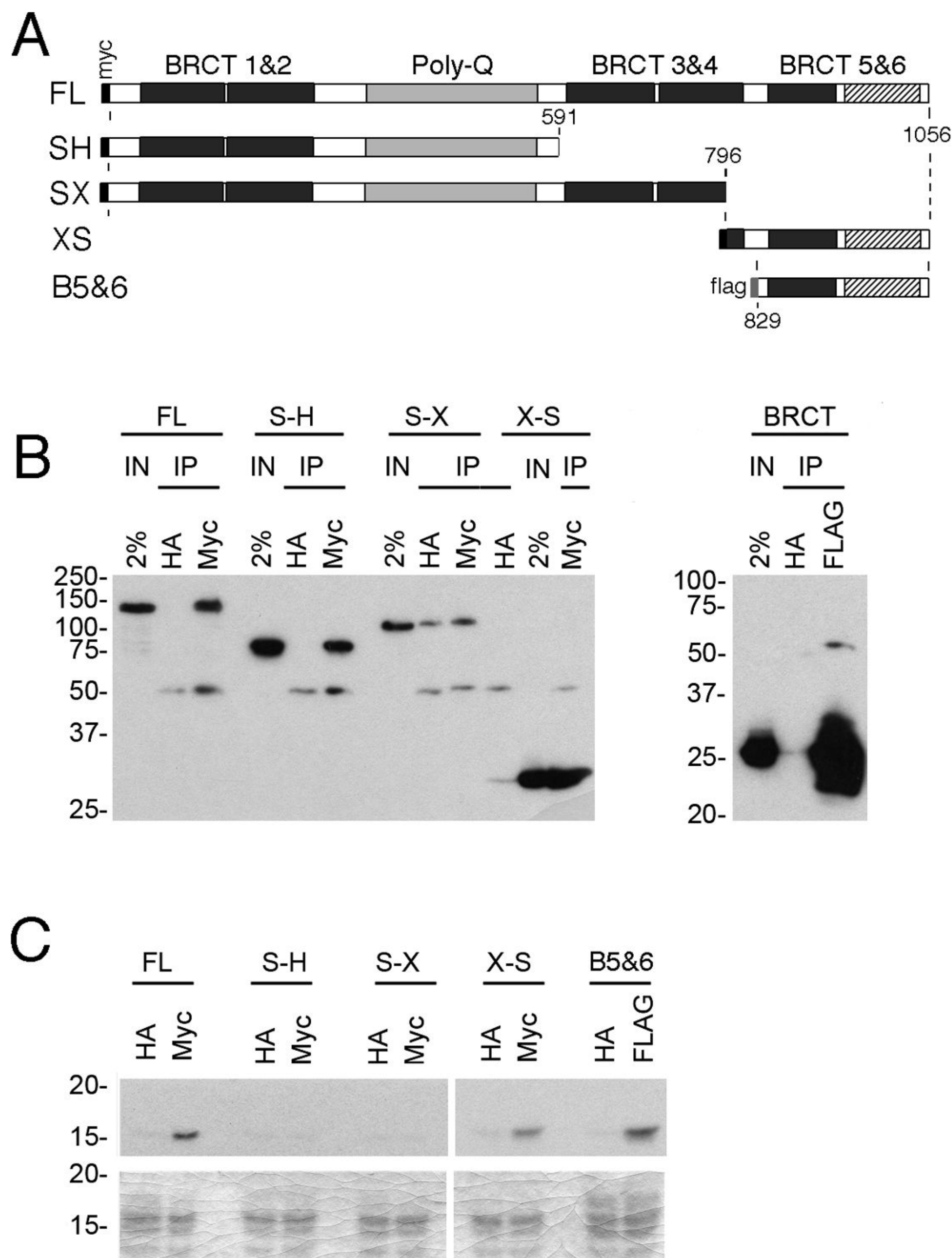

Figure 3. Deletion Analysis of PTIP

A) Schematic of full length PTIP (FL), showing the six BRCT domains and the glutamine rich region (Poly-Q). Deletions construct SH, SX, and XS contain an amino-terminal myc tag as indicated. The carboxy-terminal BRCT containing construct B5&6 contains an amino-terminal Flag epitope tag. B) Immunoprecipitates of full length and PTIP deletions used for the methylation assays. C) Immunolinked methylation assay using core histones as a substrate and [3]-SAM. Fluorogram is on top and coomasie stained gel is shown below. Note methylation of Histone H3 with full length PTIP and clones containing the BRCT domains 5 and 6. Deletions constructs SH and SX do not immunoprecipitate activity. Anti-HA is used as a control antibody.

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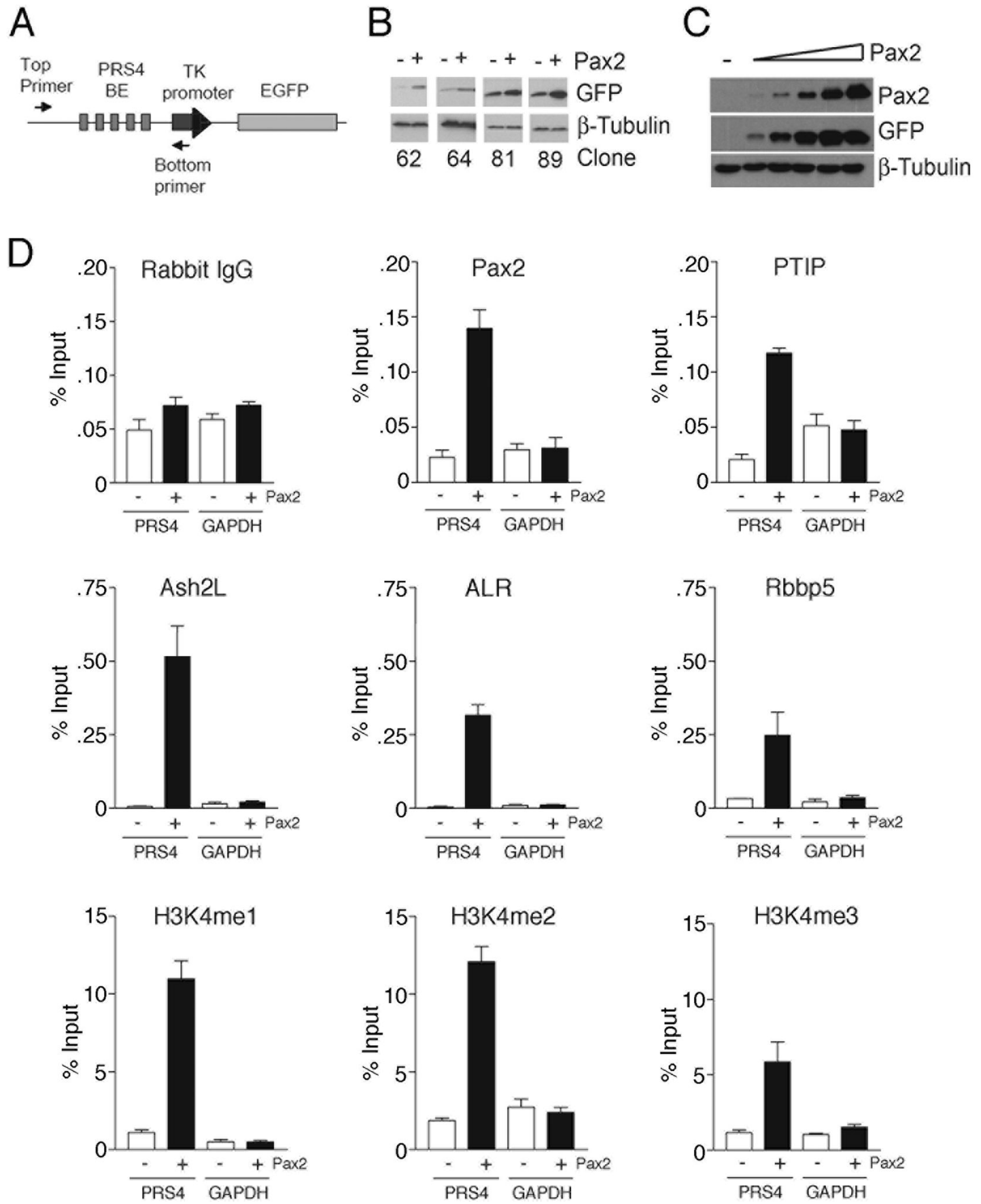

Figure 4. PTIP Interacts with a Pax2 Response Element

A) Schematic of a Pax2 integrated reporter construct in HEK293 cells. Five copies of the PRS4 binding element (BE) was inserted upstream of a minimal TK promoter driving EGFP. The primer pair used for CHIPs analysis is indicated. B) Multiple clones carrying the integrated reporter PRS4-EGFP are responsive to Pax2 transient transfection. Protein levels of EGFP and β-tubulin controls are shown. C) A dose response using increasing amounts of transiently transfected Pax2 expression plasmids. Western blots show high levels of GFP expression in response to increasing amounts of Pax2 protein. D) Chromatin Immunoprecipitation (CHIPs) from cells with or without Pax2 transfection using a variety of antibodies as indicated above each graph. Primer pairs against the Pax2 response element (PRS4) and a control promoter

(GAPDH) were used for real time quantitative PCR. Relative amounts of PCR products are expressed as a percent of input chromatin. Note that PTIP, Ash2L, ALR, and Rbbp5 are all bound to the PRS4 element in a Pax2 dependent manner. Also note the increase in histone H3K4 methylation at PRS4.

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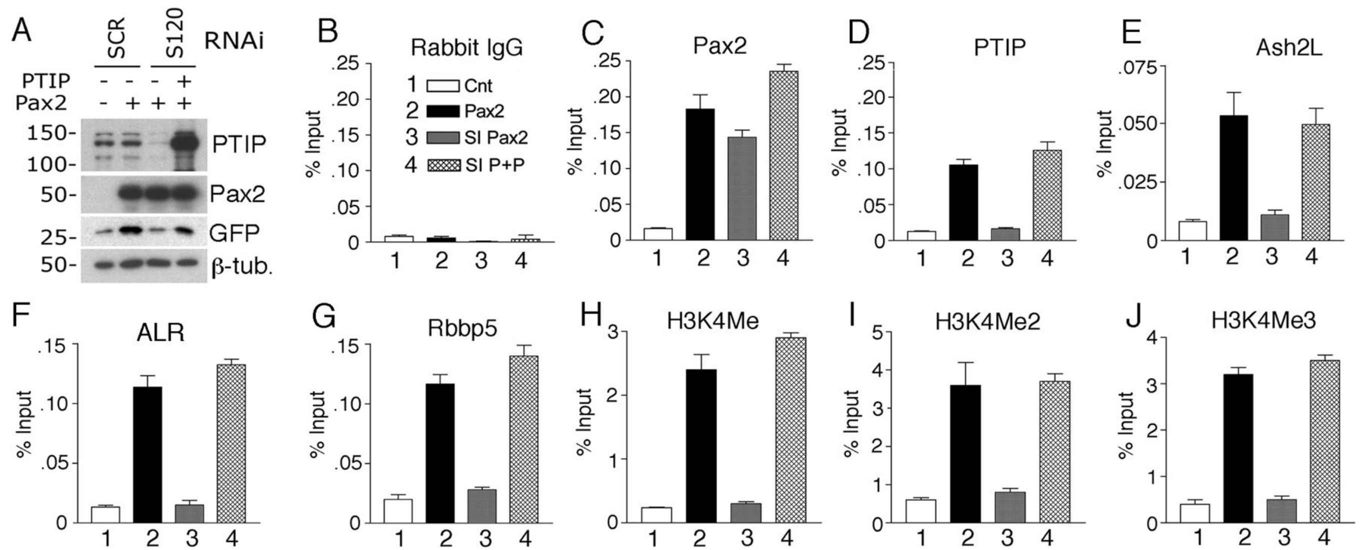

Figure 5. Assembly of the Methyltransferase complex requires PTIP

A) PTIP specific siRNAs (S120) or scrambled control siRNAs (SCR) were used to knock down PTIP in HEK293 cells containing the PRS4-EGFP reporter. Cells were transfected with Pax2 or mouse PTIP as indicated. Total protein lysates were western blotted for the proteins indicated. B-J) Chromatin immunoprecipitation assays from HEK293 cells after transfection with: 1, control vectors (Cnt); 2, Pax2 alone (Pax2); 3, siPTIP and Pax2 (SI Pax2); 4, siPTIP, Pax2, and mouse PTIP (SI P+P). The antibodies used for immunoprecipitation are indicated above the graphs. Primer pairs are against the PRS4 Pax2 binding sequence. C) Note, Pax2 binds to PRS4 independent of PTIP. D) PTIP localizes to the PRS4 site in a Pax2 dependent manner. E) Ash2L does not bind to PRS4 in the absence of PTIP. F) The SET domain protein ALR does not localize to PRS4 in the absence of PTIP. G) Similarly, Rbbp5 also requires PTIP to bind to the Pax2 recognition site. H-J) PTIP knockdown cells fail to methylate histone H3K4 at the Pax2 binding element. Exogenous, siRNA resistant PTIP can rescue this defect.

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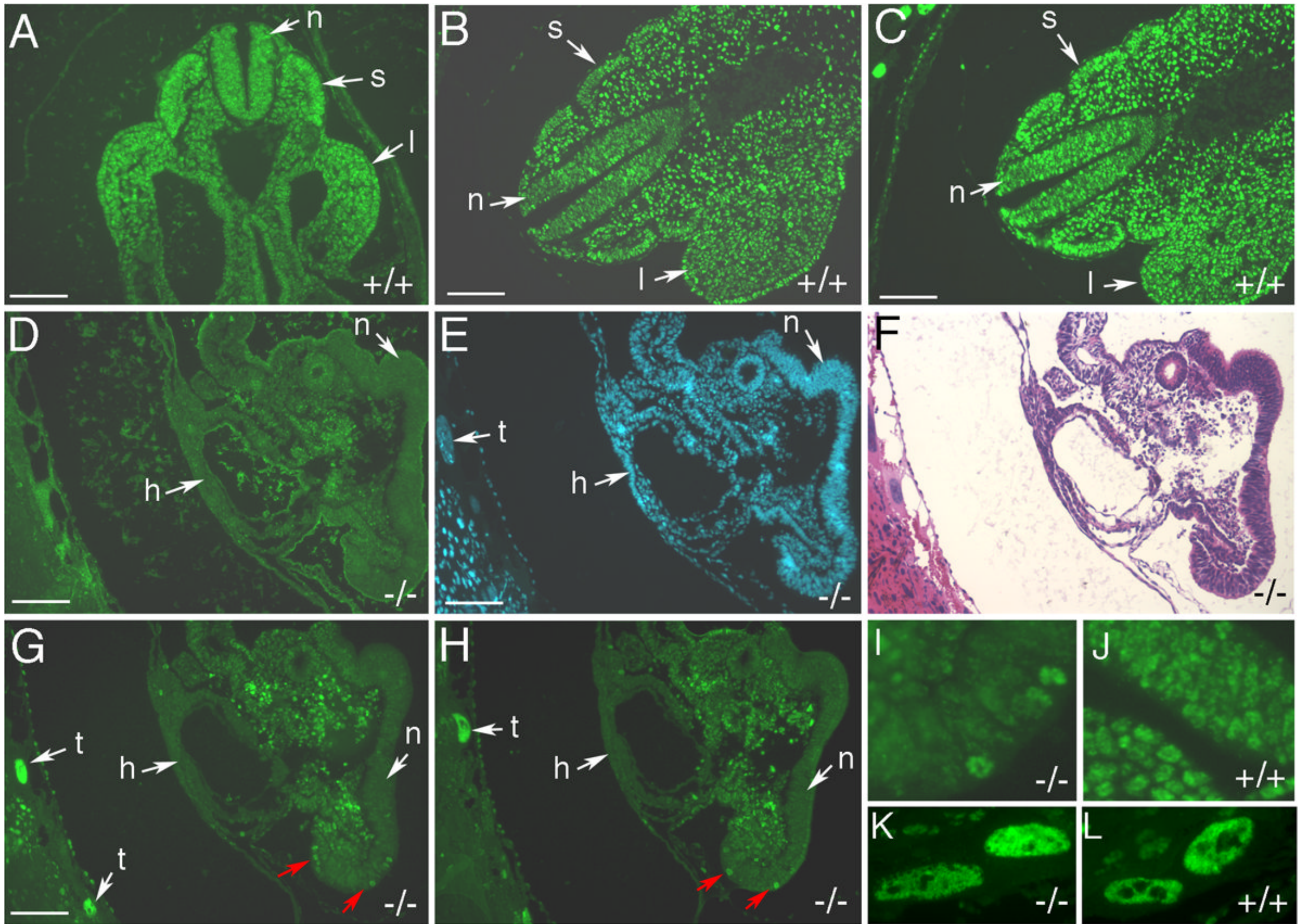

Figure 6. Immunostaining of *PTIP* Null Embryos

Littermates from E9 were fixed in paraffin, sectioned, and immunostained with antibodies against PTIP (A & D), H3K4me2 (B & G), or H3K4me3 (C, H, I-L). A) A wild-type (+/+) embryo shows strong nuclear PTIP staining. B) Strong nuclear staining is readily observed with anti-H3K4me2 antibodies in wild-type embryos in most all tissues including the neural tube (n), somites (s), and limb buds (l). C) Similar staining is observed with anti-H3K4me3 in wild-type embryos. D) $PTIP^{-/-}$ embryos show little staining with anti-PTIP in embryonic or extraembryonic tissues. E) DAPI staining of D to show nuclear integrity. F) Hematoxylin-Eosin staining of a serial section shows the disorganized head fold and presumptive bulbus cordis region of the heart. G) $PTIP^{-/-}$ embryos show reduced staining for H3K4me2 in neural tissue (n) and the presumptive heart (h), whereas the extraembryonic trophoblast nuclei (t) stain strongly. Occasional cells do exhibit nuclear staining in neural tissue (red arrows) but these are few compared to wild-type littermates. H) Similarly, $PTIP^{-/-}$ embryos show little H3K4me3 staining in the neural fold (n) and heart (h), with a few nuclei exhibiting normal levels (red arrows). I) Higher magnification of H3K4me3 staining in $PTIP^{-/-}$ neural tissue. J) Neural tube and adjacent somite of wild-type embryo stained for H3K4me3. K & L) Trophoblast nuclei of $PTIP^{-/-}$ and wild-type embryos show no difference in staining intensity for H3K4me3. All micrographs were manually set at equal exposure times. Magnification bars are 100 microns for A-H.

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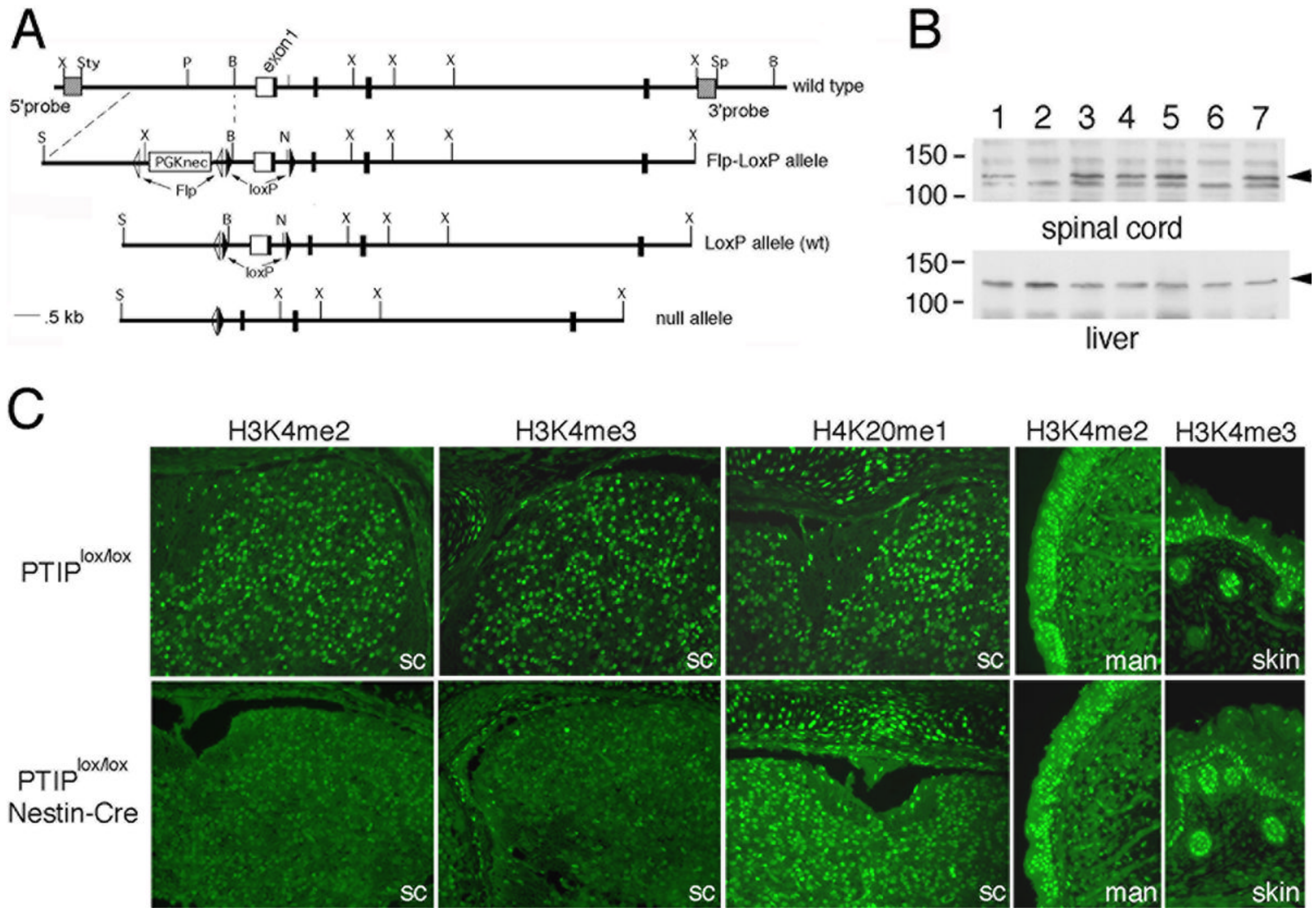

Figure 7. Conditional *PTIP* Null Allele in the Developing Spinal Cord

A) A schematic of the gene targeting strategy used to generate a floxed *PTIP* allele. LoxP sites were integrated flanking exon 1 using a PGK-neo selectable marker, which was subsequently excised with the Flp recombinase. When exposed to Cre recombinase, the LoxP allele will excise exon 1, the transcription start site, and part of the promoter region. B) Proteins isolated from E17.5 spinal cord and liver were assayed for PTIP by western blotting. Lanes 2 and 6 are lysates from $PTIP^{loxP/loxP}$ mice that also carried the Nestin-Cre transgene, note reduced levels of PTIP protein in spinal cord but not in liver. Blots were overexposed to show equal loading. C) Immunostaining of E17.5 embryos with antibodies against modified histones as indicated. The tissues shown are spinal cord (sc), the mandibular region (man), and the skin and hair follicles. In $PTIP^{loxP/loxP}$ embryos, strong nuclear staining is seen with all antibodies. However mice also carrying the Nestin-Cre transgene show reduced levels of staining for H3K4me2 and H3K4me3 in the spinal cord but not in other tissues. Staining for H4K20me1 was similar, regardless of PTIP expression.

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Table 1

Mass Spectrometry of PTIP Interacting Proteins

Protein	No. of Pep	PTIP coverage	No. of Pep	PTIP (ir) coverage
ALR	65	19%	50	13%
MLL3	57	18%	63	19%
53BP1	4	3%	72	47%
UTX	41	37%	31	24%
SMC1	23	23%	20	21%
Rad50	22	26%	22	26%
Ash2L	35	27%	41	30%
Rbbp5	28	78%	19	71%
WDR5	5	15%	9	21%