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Physical interaction between MPP8 and PRC1 complex and its implication for regulation of spermatogenesis

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ABSTRACT

Epigenetic modifications such as DNA methylation and histone H3 lysine 27 methylation (H3K27me) are repressive marks that silence gene expression. The M phase phosphoprotein (MPP8) associates with proteins involved in both DNA methylation and histone modifications, and therefore, is a potential candidate to mediate crosstalk between repressive epigenetic pathways. Here, by performing immunohistochemical analyses we demonstrate that MPP8 is expressed in the rodent testis, especially in spermatocytes, suggesting a role in spermatogenesis. Interestingly, we found that MPP8 physically interacts with PRC1 (Polycomb Repressive Complex 1) components which are known to possess essential function in testis development by modulating monoubiquitination of Histone H2A (uH2A) and trimethylation of Histone H3 Lysine 27 (H3K27me3) residues. Knockdown analysis of MPP8 in HeLa cells resulted in derepression of a set of genes that are normally expressed in spermatogonia, spermatids and mature sperm, thereby indicating a role for this molecule in silencing testis-related genes in somatic cells. In addition, depletion of MPP8 in murine ES cells specifically induced expression of genes involved in mesoderm differentiation, such as Cdx2 and Brachyury even in the presence of LIF, which implicated that MPP8 might be required to repress differentiation associated genes during early development. Taken together, our results indicate that MPP8 could have a role for silencing genes that are associated with differentiation of the testis and the mesoderm by interacting with epigenetic repressors modules such as the PRC1 complex.

1. Introduction

The M phase phosphoprotein 8 (MPP8) was identified as a protein that is highly phosphorylated during M phase [1]. MPP8 possesses a chromodomain in the amino terminal region which has an affinity for trimethylated histone H3 lysine 9 (H3K9me3) residues [5,6]. This chromodomain also binds to dimethylated Dnmt3a (K44me2), a mammalian *de novo* DNA methyltransferase, and auto-methylated GLP or G9a, both of which are linked with H3K9 dimethylation [2]. Given that the MPP8 chromodomain forms a dimmer in solution [3], it is possible that this protein could play a role as a scaffold to form a multimeric silencing complex comprising of Dnmt3a-MPP8-GLP/G9a to regulate chromatin templates [2]. During cell cycle progression, MPP8 predominantly localizes to chromatin, but dissociates during interphase and early mitosis, respectively, although its expression appeared to be constant [7]. Chromatin dissociation of MPP8 during M phase is regulated by at least in part by cyclin B1-Cdk1-dependent phosphorylation [7]. Importantly, tissue-specific expression of MPP8 in mice revealed a predominant expression pattern in testis and far lower ubiquitous expression in most of the tissues, suggesting that this protein could have a role during spermatogenesis.

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Polycomb group (PcG) proteins are evolutionary conserved complexes from flies to mammal that regulate body segmentation through the modulation of higher order chromatin structure [8]. In mammals, PcG proteins form at least two distinct repressive protein complexes, PRC1 and PRC2, that catalyze histone modifications such as ubiquitylation of H2A at K119 and methylation of histone H3 at K27, respectively [9]. PcG complexes are critical components of the molecular memory that maintain gene expression patterns beyond cell proliferation and PcG-mediated gene silencing is essential for maintaining stemness in various types of stem cells through suppressing expression of genes determining differentiation phenotypes [10,11]. Notably, mice lacking Scmh1, cbx2, and those lacking both Ring1B and Ring1A demonstrated severe defects in testis development, suggesting roles of PRC1 in spermatogenesis [12–15].

Spermatogenesis is a complex and coordinated process for cellular differentiation and is essential for sexual reproduction [16]. The development of male germ cell is performed through intricate and highly ordered sequential steps including meiosis, genetic recombination, haploid gene expression, acrosome and flagellum formation, chromatin remodeling and condensation [17]. These processes should therefore require a precise and stringently controlled program for chromatin reorganization. Like any other differentiating cells, the characteristics of the individual cell types that comprise the testis are dependent on differential gene expression including testis-specific genes or isoforms, which can be successfully achieved through complex networks of epigenetic regulation and interactions between transcription factors [18].

In order to address the physiological function of MPP8 during spermatogenesis, we performed immunohistochemical staining in the testis and found that it localized predominantly in spermatocytes and modestly in spermatogonia, while weak or very faint staining could be observed in spermatids and mature sperm. Importantly, MPP8 physically interacted with Ring1B and Bmi1, components of PRC1 and knockdown analysis in HeLa cells revealed that MPP8 is required for silencing a group of genes that are normally expressed in spermatogonia and spermatids. Depletion of MPP8 in ES cells specifically induced expression of genes involved in mesoderm differentiation, such as Cdx2 and Brachyury. Thus, our results suggest a previously unidentified role for MPP8 in testis development and spermatogenesis by regulation of testis-specific gene expression, possibly through modulating PRC1 function.

2. Materials and methods

2.1. Yeast two-hybrid screening

The *pGBKT7-MPP8* plasmid was generated by insertion of the full-length human MPP8-encoding sequence. *pGBKT7-MPP8* was transformed into the yeast strain AH101 and mated with yeast Y187 pretransformed with a HeLa cell cDNA library (BD biosciences).

2.2. Immunohistochemistry of rat testis sections

The testes, heart, brain, liver, small intestine, ovary, and endometrium of F344 wild type rat were removed and submerged into 10% (v/v) neutral buffered formalin for 6 h, embedded in paraffin and sectioned at a thickness of 3 μm.

Immunohistochemical staining was applied on paraffin-embedded sections (3 mm thick) of testes and multiple organs of F344 rats. Immunostaining was performed on a VENTANA DISCOVERY HX auto-immunostainer (Roche, Mannheim, Germany). Antigen retrieval was carried out with CC1 buffer (Roche) for 60 min at 100 °C. Sections were incubated for 60 min with antibodies specific for primary antibodies and sequentially with secondary antibodies and avidin–biotin complex (Vectastatin Elite ABC kit; Vector Laboratory, Burlingame, CA), then binding sites were visualized with diamino-benzidine (DAB; Sigma). Sections were lightly counterstained with hematoxylin to facilitate microscopic analysis. Microscope images were obtained using Olympus AX70 (Olympus, Tokyo, Japan).

2.3. Cell culture

HeLa cells were cultured in DMEM supplemented with 10% FBS. E14 mES cells were cultured on 0.1% gelatin-coated plates in DMEM supplemented with 15% FBS, 1 mM Sodium Pyruvate (Gibco), 1 × MEM non-essential amino acid (Gibco) and 10^3units ESGRO mouse LIF medium supplement (Millipore). All cells were cultured at 37 °C under 5% CO_2.

2.4. Lentiviral transduction

Lentivirus expressing the MPP8 shRNA (GCAACAGATGCAATTCCAAGT) was generated by the co-transfection of 293T cells with pCMV-VSV-G-RSV-RevB (a gift from H. Miyoshi), pCAG-HIVgp (also a gift from H. Miyoshi), and the respective CSIV-TRE-RfA-UbC-KT using the calcium phosphate co-precipitation method. Cells infected with viruses were treated with 10 μg/ml Blasticidin for 3 days. To knockdown of MPP8, Doxycycline (Sigma–Aldrich) was added to the medium at the concentration of 1 μg/ml.

2.5. Nuclear protein extraction, immunoprecipitation and immunoblotting

Nuclear protein extraction for immunoprecipitation, HeLa cells were grown to ≤80% confluence, trypsinized, and centrifuged (200× *g* for 3 min at room temperature), then washed in ice cold PBS. Protein was prepared as described previously [19]. Immunoprecipitation, nuclear extracts were diluted with buffer D (20 mM Hepes-KOH, pH 7.9, 100 mM KCl, 0.2 mM EDTA, 20% glycerol) and incubated with 1 μg of primary antibodies at 4 °C for overnight and incubated with 10 μl of protein A-agarose (Roche) at 4 °C for 1 h Beads were thoroughly washed three times with buffer D and resuspended in 1 × SDS sample buffer and boiled 100 °C for 5 min. Sample was analyzed by immunoblotting.

2.6. Antibodies

The antibodies used in this study were MPP8 (16796-1-AP, proteintech group), Bmi1 (D20B7, Cell signaling), Flag (F3165, sigma), Ring1B (for immunoblotting, 39663, active motif and for immunoprecipitation, ab101273, abcam).

2.7. Microarray experiment

HeLa cells infected shMPP8 were cultured with/without Doxycycline for 3 days and total RNA was prepared using ISOGENII (Nippongene). RNA hybridization, wash and analysis were performed using SurePrint G3 Human GE 8×60k Ver2.0 (Agilent Technologies), GeneSpring GX Ver12.6.0 (Agilent Technologies). Microarray expression profiling was performed by Oncomics (Nagoya, Japan).

2.8. Data analysis

Tests for gene ontology (GO) and tissue enrichment patterns were performed using DAVID (the Database for Annotation,

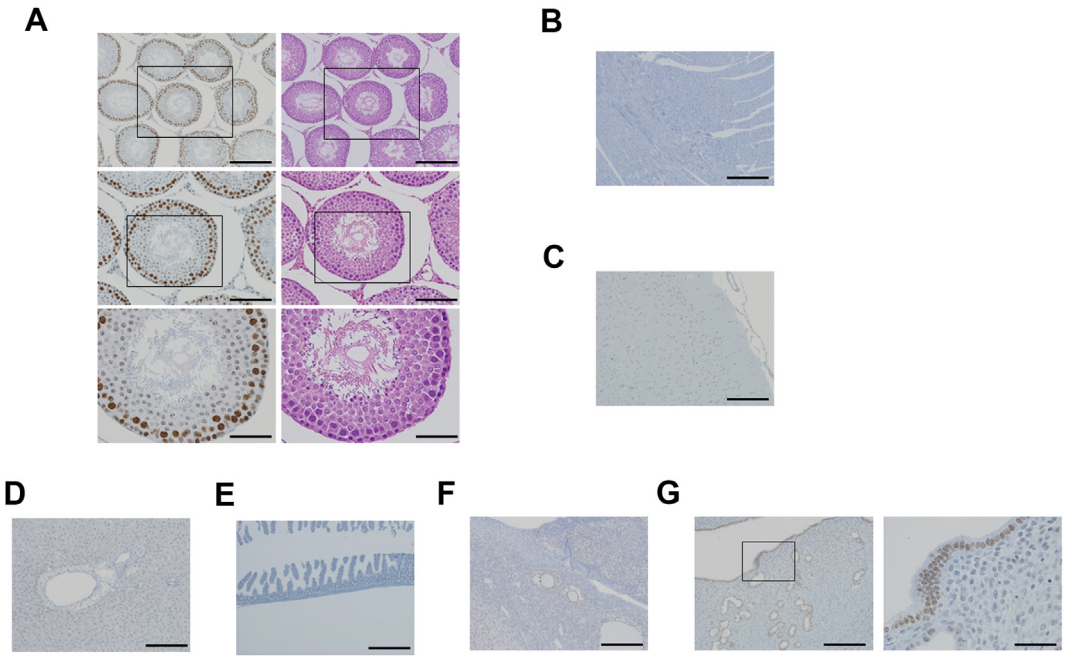

Fig. 1. MPP8 is predominantly expressed in spermatocytes and modestly in spermatogonia.
Immunohistochemical staining of paraffin-embedded rat testis (A), heart (B), brain (C), liver (D), small intestine (E), ovary (F) and endometrium (G) using Anti-MPP8. HE staining was also shown in (A). Bar = 200 μm in (A) top panel, (B)–(F) and (G) left panel, 100 μm in (A) middle panel, 50 μm in (A) bottom panel and (G) right panel.

Visualization and Integrated Discovery; http://www.david.abcc.ncifcrf.gov/) web resources. ChIP-seq data for RNA polymerase II (Pol-II), H3K27me3, H3K4me3 and H3K9me3 enrichment in K562 cells were obtained from the publicly available GEO database (gene expression omnibus, http://www.ncbi.nlm.nih.gov/geo/). These datasets were deposited by Bernstein et al. (MGH, Harvard) and the accession number for each ChIP-seq experiment is shown in Fig. 3C. ChIP-seq data were visualized with the IGV (integrative genomics viewer) browser distributed by Broad Institute (http://www.broadinstitute.org/igv/). RNA-seq datasets for testicular cells (spermatogonia: Sg, spermatocytes: Sc; spermatids: Std; spermatozoa: Sz) deposited by Soumillon et al. [20] were retrieved from the GEO database (series accession number GSE43717). University of Toronto BAR (Bio-Analytic Resource, http://www. bar.utoronto.ca/) tools were used for heatmap analysis.

2.9. RT-qPCR assay

Tet-inducible shRNA transfected E14 mES cells were cultured with 1 μg/ml doxycycline with presence of LIF medium for 7 days. RNA was extracted from E14 mES cells with IsogenII (Nippon Gene). 5 μg of total RNA were converted to cDNA using ReverTra Ace qPCR RT kit (TOYOBO). cDNA levels were assayed by real-time PCR with FastStart Universal SYBR Green Master (Roche) and analyzed on real time PCR ABI7500fast (Applied Biosystems). PCR was performed using following primers:

Cdx2 F (5′-AAGACAAATACCGGGTGGTG-3′),
Cdx2 R (5′-CCAGCTCACTTTTCCTCCTG-3′),
Brachyury F (5′-TCCCGAGACCCAGTTCATAG-3′),
Brachyury R (5′-TTCTTTGGCATCAAGGAAGG-3′),
Arbp F (5′-CAAAGCTGAAGCAAAGGAAGAG-3′), and
Arbp R (5′-AATTAAGCAGGCTGACTTGGTTG-3′).

The expression of individual genes was normalized to the level of Arbp.

3. Results and discussion

3.1. MPP8 is predominantly expressed in spermatocytes and modestly in spermatogonia

Immunohistochemical analysis in rat testis using specific antibodies to MPP8 revealed robust staining in spermatocytes and spermatogonia, while the staining faint in spermatid, and sperm (Fig. 1A). Tissue-specific immunohistochemical analyses demonstrating hardly detectable levels of MPP8 in heart (Fig. 1B), brain (Fig. 1C), liver (Fig. 1D), small intestine (Fig. 1E), ovary (Fig. 1F), and endometrium (Fig. 1G). These results were consistent with our observations from tissue-specific quantitative real-time PCR analyses which showed that MPP8 is mostly expressed in testis [7]. Taken together, these results suggested that MPP8 is predominantly and modestly expressed in spermatocytes and spermatogonia

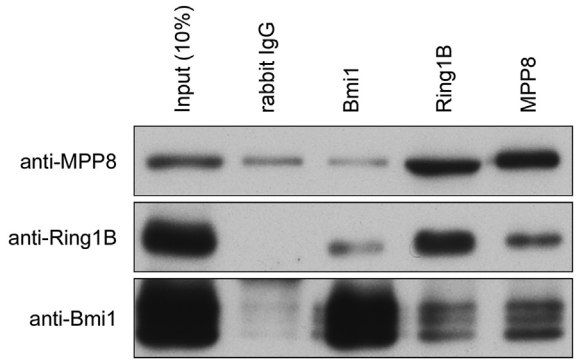

Fig. 2. MPP8 physically interacts with PRC1 components, Ring1B and Bmi1. Nuclear extracts prepared from HeLa cells were immunoprecipitated with the indicated antibodies. The resultant immunoprecipitates were subjected to immunoblotting using the indicate antibodies.

A

Term	Count	%	P-Value	Fold Enrichment	FDR
Brain cortex	6	1.5	6.20E-02	2.8	5.40E+01
Keratinocyte	6	1.5	6.90E-02	2.7	5.80E+01
Testis	84	20.5	8.30E-02	1.2	6.50E+01
Hippocampus	13	3.2	9.10E-02	1.7	6.80E+01

Tissue-wise enrichment of upregulated genes upon MPP8 knockdown

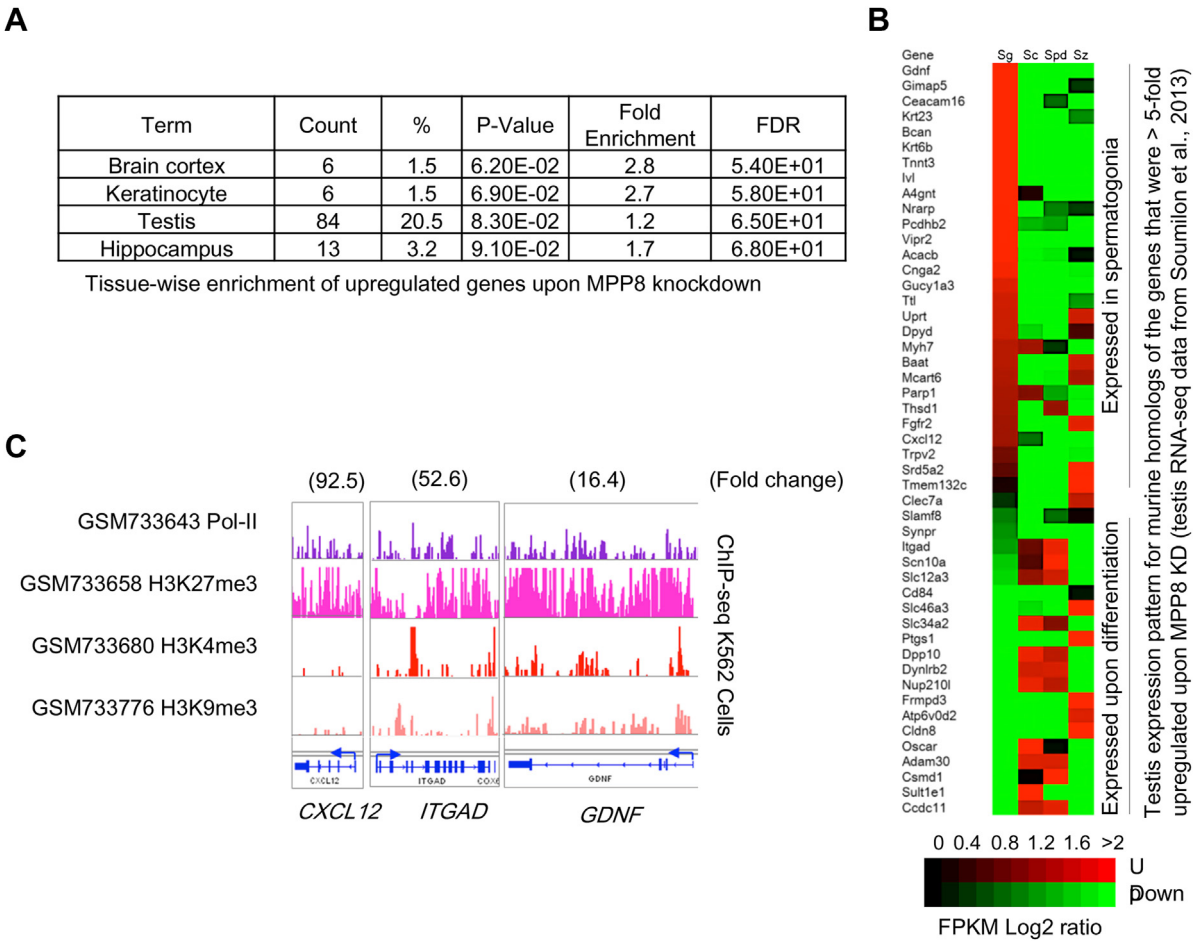

Fig. 3. MPP8 represses genes that are transcribed in spermatogonia, spermatids and spermatozoa. (A) Tissue enrichment patterns for upregulated genes were analyzed. The table shows the list of enriched tissues, number (count) and percentage of genes (%), p-value, fold enrichment and FDR. (B) RNA-seq (FPKM) expression patterns of upregulated (>5-fold) genes. The types of testicular cells are delineated as Sg (spermatogonia), Sc (spermatocytes), Std (spermatids) and Sz (spermatozoa). Fold expression levels (compared to the median, Log2 values) are represented in a heatmap. The color scale of the heatmap is given at the bottom. (C) ChIP-seq profiles for RNA polymerase II (Pol-II), H3K27me3, H3K4me3, and H3K9me3 are exhibited for representative genes that were found to be upregulated upon MPP8 KD. The GEO accession number of each dataset is shown at the left. Gene names are given at the bottom of each panel and the fold upregulation (upon MPP8 KD) are shown at the top.

respectively and therefore could be involved in regulation of spermatogenesis.

3.2. MPP8 physically interacts with PRC1 components

In order to determine the physiological relevance of MPP8 in spermatogenesis, we performed yeast two-hybrid screening using MPP8 as a bait. Of a total of 5×10^6 transformants from a HeLa cell cDNA library, 27 positive colonies were confirmed to be lacZ-positive. They contained overlapping several cDNAs, such as genes derived from MBD4, WBP5, and Ring1B (Table 1). Even among them, we will focus on Ring1B, a component of PRC1 complexes, because PRC1 was reported to express most highly in testis and to be involved in spermatogenesis [13]. Physical interaction of MPP8 with Ring1B was confirmed by IP-western blotting analysis. As shown in Fig. 2, Ring1B was readily detected in MPP8 immunoprecipitates from HeLa cells. Interestingly, Bmi1, another component of PRC1, was also detectable in MPP8 immunoprecipitates, suggesting that MPP8 forms a complex with PRC1 complex. Consistent with this, MPP8 was also detected in Ring1B immunoprecipitates. Taken together, these results indicate that MPP8 appears to form a stable complex with PRC1.

Table 1
The isolated genes by two-hybrid screening using human MPP8 as bait.

Gene	RefSeq ID	Function	Clone number
WBP5	NM_001006612.1	WW domain binding	4
MBD4	NM_001276270.1	DNA repair and DNA demethylation	3
Ring1B	NM_007212.3	Transcriptional regulation, ubiquitin ligase	3
FAM968		Unknown	2
v-myc		Transcriptional regulation	2
c-myc	NM_002467.4	Transcriptional regulation	1
CCDC34	NM_030771.1	Unknown	1
CHRAC1	NM_017444.5	Histone-fold protein	1
GRAMD4	NM_015124.3	Mitochondrial effector of E2F1-induced apoptosis	1
HINT2	NM_032593.2	Nucleotide hydrolases	1
ISCA2	NM_001272007.1	Metal ion binding	1
MBD1	NM_001204136.1	Transcriptional regulation	1
NSD1	NM_022455.4	H3-K36-HMTase, H4-K20-HMTase	1
SAP30BP	NM_001301839.1	Positive regulation of cell death	1
SKP1	NM_006930.3	The component of SCF complexes	1
SPCS2	NM_014752.2	Peptidase activity	1
USP8	NM_001128610.2	Ubiquitin-specific protease activity	1
WWC1	NM_001161661.1	Cytoplasmic phosphoprotein that interacts with PRKC-zeta and dynein light chain-1	1

3.3. Depletion of MPP8 in HeLa cells resulted in transcriptional activation of genes that are normally expressed in spermatogonia

To elucidate the function of MPP8 in global gene regulation, we performed microarray analyses in HeLa cells in which the MPP8 gene was knocked down by shRNAs. Microarray results revealed that approximately 800 and 500 genes were upregulated or downregulated (by > 2-fold), respectively, upon MPP8 knockdown (KD). To gain insight into the role of the genes that were upregulated after MPP8 KD, we performed functional annotation of the upregulated genes by using the DAVID (Visualization and Integrated Discovery) tools and found that many of these genes (84 annotations) associated with the "testis" tissue category, supporting our aforementioned findings that MPP8 could have a role in testis (Fig. 3A).

We went on to explore the link between MPP8 and testis-specific gene expression by analyzing publicly available datasets (#GSE43717) [20] for gene expression level in various testicular cells such as spermatogonia (Sg), spermatocytes (Sc), spermatids (Std) and spermatozoa (Sz) measured by deep sequencing (RNA-seq). We isolated the genes that were upregulated by 5-fold upon MPP8 KD in HeLa cells and extracted their murine counterparts by homology search (for example the murine Parp1 is a homolog of human PARP1). We then examined the relative expression level of these genes in Sg, Sc, Std and Sz cells using the RNA-seq data. The results are shown in a heatmap in Fig. 4B. Interestingly, we observed that the genes that were upregulated by MPP8 KD, were mainly associated with non-spermatocyte cells such as spermatogonia (Sg), spermatids (Std) and spermatozoa (Sz). For example, the Gdnf (Glial cell-derived neurotrophic factor) gene is normally expressed predominantly in the spermatogonia but not in other cell types in the testis. Similarly, the Thsd1 (thrombospondin, type I, domain containing 1) gene is strongly expressed in the spermatids (Std), while factors such as Ptgs1 (prostaglandin-endoperoxide synthase 1) exhibits a spermatozoa specific transcription pattern. These findings implicate that MPP8 might have a function to silence these genes in spermatocytes, presumably by cooperating with other factors. Intriguingly, an opposite pattern was also observed for some genes such as Sult1e1 (sulfotransferase family 1E, estrogen-preferring, member 1) and Parp1 (poly (ADP-ribose) polymerase 1), which showed enrichment in spermatocytes. It is conceivable that some of the spermatocyte expressed genes are maintained in a moderate transcriptional state under physiological conditions by MPP8 mediated repression. In the absence of MPP8, these genes were therefore more strongly expressed.

Further, to understand the link between PRC1 and MPP8, we analyzed the publicly available ChIP-seq datasets for H3K27me3, H3K4me3, H3K9me3 and RNA Pol-II deposition in the human K562 cell line. In general, we observed frequent enrichment of H3K27me3 in the genes that were upregulated upon MPP8 KD such as CXCL12, ITGAD and GDNF (Fig. 3C, fold upregulation upon MPP8 KD is shown at the top of each panel). Importantly, we did not observe a strong enrichment of H3K9me3 marks in these loci. In mammals, H3K27me3 modification is mediated by the PcG proteins [21]. Association of H3K27me3 marks with the gene loci that are derepressed upon MPP8 knockdown could be therefore linked by a putative PRC1-dependent mechanism. Our gene ontology tests and tissue enrichment examinations have indicated that MPP8 could have a role for silencing differentiation associated factors (e.g. neural genes, and genes that are expressed in differentiated spermatid cells). Polycomb factors are well-known as evolutionarily conserved modules for silencing differentiation associated genes among species [21]. We analyzed publicly available dataset of Ring1B bound regions and found that Ring1B associated with 70 genes that were up-regulated upon MPP8 knockdown, such as Gdnf and Gimap5 (series accession number GSE41316) [22]. Using a database of Ring1B knockout gene expression profile (series accession number GSE10476) [23], we also identified 9 genes, such as HMGA2 and NT5E, which were up-regulated upon MPP8 knockdown as well as Ring1B knockout cells. Taken together, these

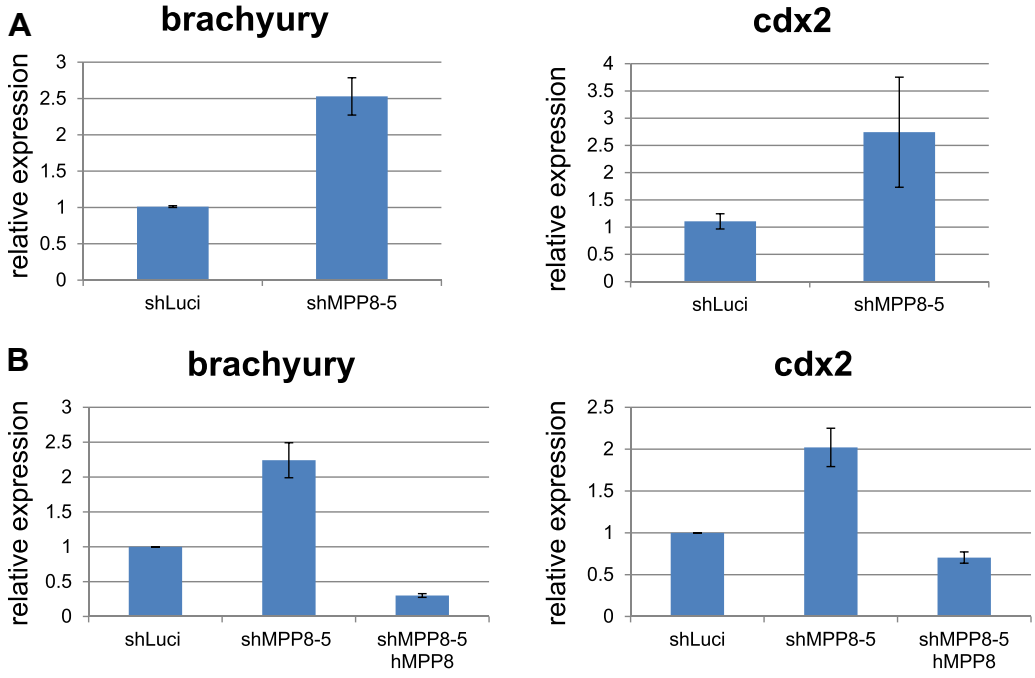

Fig. 4. Depletion of MPP8 in ES cells specifically induce the expression of mesoderm markers. (A) Mouse ES cells were infected with Tet-on inducible lentiviruses expressing shControl or shMPP8. Infected cells were cultured with Dox for 7 days in the presence of LIF. (B) Mouse ES cells expressing Tet-on inducible shControl, shMPP8 and shMPP8 and shRNA resistant human MPP8 were cultured in the presence of Dox for 7 days. Total RNAs prepared from cells from (A) and (B) were subjected to quantitative real-time RT-PCR using a set of primers within the indicated marker genes involved in pluripotency and those involved in germ-layer differentiation.

data suggests a putative association between MPP8 and the PRC1 complex.

3.4. Depletion of MPP8 in ES cells resulted in transcriptional activation of genes involved in mesoderm induction

Given that PRC1 function is essential for maintenance of pluripotency in ES cells, we speculated that it is also the case with MPP8. Thus, we examined whether depletion of MPP8 in ES cells could affect the expression of marker genes involved in germ-layer differentiation. When MPP8 was depleted from ES cells in the presence of LIF, quantitative PCR analysis revealed the specific and significant induction of mesoderm markers, such as Brachyury and Cdx2 (Fig. 4A). Importantly, increased expressions of mesoderm markers were effectively suppressed when wild-type MPP8 was ectopically expressed in endogenous MPP8-depleted ES cells (Fig. 4B), confirming the specificity of its repressive effect on the expression of mesoderm markers. Since genital organs including testis as well as hematopoietic organs and nervous systems, whose development was regulated by PRC1 complexes, were derived from mesoderm, MPP8 appears to be involved in mesoderm induction, testis development and spermatogenesis through suppression of genes specifically involved in these processes, possibly under the collaboration with PRC1 complexes.

Conflict of interest

None.

Acknowledgments

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Transparency document

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