The mixed lineage leukemia 4 (MLL4) methyltransferase complex is involved in transforming growth factor beta (TGF-β)-activated gene transcription

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ABSTRACT
Sma and Mad related (SMAD)-mediated Transforming Growth Factor β (TGF-β) and Bone Morphogenetic Protein (BMP) signaling is required for various cellular processes. The activated heterotrimeric SMAD protein complexes associate with nuclear proteins such as the histone acetyltransferases p300, PCAF and the Mixed Lineage Leukemia 4 (MLL4) subunit Pax Transactivation domain-Interacting Protein (PTIP) to regulate gene transcription.

We investigated the functional role of PTIP and PTIP Interacting protein 1 (PA1) in relation to TGF-β-activated SMAD signaling. We immunoprecipitated PTIP and PA1 with all SMAD family members to identify the TGF-β and not BMP-specific SMADs as interacting proteins. Gene silencing experiments of MLL4 and the subunits PA1 and PTIP confirmed TGF-β-specific genes to be regulated by the MLL4 complex, which links TGF-β signaling to transcription regulation by the MLL4 methyltransferase complex.

KEYWORDS
transcription regulation; SMAD transcription factor; transforming growth factor beta (TGF-β); bone morphogenetic protein (BMP); histone methylation; mixed lineage leukemia (MLL)

Introduction
Transforming Growth Factor β (TGF-β) and Bone Morphogenetic Protein (BMP) signal transduction routes in the cell are implicated in various cellular processes. Both signaling pathways start with the activation of a transmembrane receptor pair located at the plasma membrane. Receptor activation causes phosphorylation of downstream receptor-activated proteins called Sma and Mad (SMADs). These receptor-activated SMADs (R-SMADs) in turn bind to SMAD4 as a heterotrimeric protein complex to regulate gene transcription. Depending on the activating signaling ligand, different R-SMAD proteins become activated. BMP causes phosphorylation of SMAD1/5/9, while TGF-β causes phosphorylation of SMAD2/3. The SMAD-mediated signaling routes are regulated by the SMAD6 and SMAD7 as negative regulators.

Activated SMAD complexes are able to interact with transcription factors and co-localize to specific genomic locations. Furthermore, SMAD complexes bind to the coactivator complex p300 and the PCAF histone acetyltransferases. Recruitment of p300 or the PCAF subunit of SAGA and ATAC complexes stimulates gene transcription via the acetylation of histone tails in proximity of the complex. In addition, proteins containing double bromodomains or certain plant homeodomain (PHD) fingers recognize and bind acetylated histones.

Other histone modifications that are associated with active gene transcription are H3K4 mono- and trimethylation. H3K4 trimethylation (H3K4me3) is present on the promoters of active genes and is a binding site for the TFIID subunit TAF3. H3K4 monomethylation (H3K4me1) is found at active enhancer sites. H3K4 methylation is carried out by the six SET1/MLL methyltransferase complex types present in human cells. SET domain-containing protein 1A (SET1A) and SET1B complexes are responsible for the bulk of H3K4 trimethylation while Mixed Lineage Leukemia 3 (MLL3, also known lysine methyl transferase 2C (KMT2C)) and MLL4 (KMT2D)
complexes are maintaining H3K4 mono-methylation at enhancer sites.\textsuperscript{16,17} The SET1/MLL complexes all contain the WDR5, RBBP5, ASH2L and DPY30 proteins that make up the common WRAD core.\textsuperscript{18} In addition to the WRAD core, the MLL3 and MLL4 complexes are composed of Pax Transactivation domain-Interacting Protein (PTIP) and PTIP Interacting protein 1 (PA1).\textsuperscript{18-20} Apart from interacting in the MLL3 and MLL4 methyltransferases, PTIP and PA1 also form a distinct protein complex that can exist and act independently during DNA damage and IgH class switching.\textsuperscript{18,21,22} SMAD2 was previously identified as an interaction partner of PTIP.\textsuperscript{18,23} However, the link to the MLL3 and MLL4 methyltransferase complex has not been clarified yet.

Here, we report the relationship between SMAD-mediated signaling and the gene regulatory function of PTIP. In a comprehensive approach, we determined the interaction of PTIP and PA1 proteins with all SMADs to identify a link between the TGF-β SMADs and PTIP and PA1. Gene expression analysis following PTIP and PA1 knockdown confirms PTIP and PA1 to be involved in TGF-β gene activation. Expression analysis after MLL3 and MLL4 knockdown shows this activation to be MLL4 but not MLL3 dependent. This links SMAD signaling to the MLL4 methyltransferase complex.

**Results**

**PTIP and PA1 bind to TGF-β activated SMADs** – The interaction of PTIP and SMAD2 has been identified and studied before\textsuperscript{23} and was confirmed previously by us using an unbiased proteomics approach.\textsuperscript{18} We hypothesized that other SMAD family members could interact with PTIP. To systematically identify the SMAD proteins binding to PTIP, HEK 293T cells were co-transfected with HA-tagged SMAD and GFP-PTIP expression plasmids and affinity purification was performed via the GFP-tag of GFP-PTIP. Immunoblotting with the HA-antibody revealed the presence of TGF-β activated SMAD2, 3 and 4 in the GFP purifications, while BMP stimulated SMAD1 and 5 were absent (Fig. 1A). We have previously demonstrated that these SMAD1 and 5 expression constructs result in functional SMAD proteins.\textsuperscript{24} Interestingly, SMAD9 was also present at low levels. PTIP and PA1 are subunits of the MLL3 and MLL4 methyltransferase complexes. Additionally, PTIP and PA1 can form independent heterodimer complexes. To examine the interaction of SMADs with PA1, HEK 293T cells were similarly co-transfected with HA-SMADs and GFP-PA1. GFP-affinity purification on the transfected cell lysates also showed interaction with the TGF-β SMADs (Fig. 1B). Interestingly, SMAD6 and SMAD7 also bind to PA1, but this binding is not observed with PTIP. In addition, SMAD9 was again precipitated at low levels. Overall, these experiments indicate that the TGF-β SMADs but not the BMP SMADs interact directly with PTIP and PA1.

**Investigation of TGF-β and BMP target genes in U-2 OS** – Previous studies have identified TGF-β and BMP signaling target genes in cell systems like the HaCaT keratinocytes and osteoblast BMP inducible C2C12 myoblast cell lines.\textsuperscript{25-28} The human osteosarcoma derived cell line U-2 OS is easily transfectable and efficient siRNA-mediated knockdown of gene expression can be obtained.\textsuperscript{29} To investigate a functional link of TGFβ with PTIP and PA1, mRNA expression of known TGF-β and BMP target genes was tested for TGF-β or BMP sensitivity in U-2 OS cells (Fig. 2A and 2B). TGF-β treatment increased mRNA expression of the SERPINE1, PMEPA1, SKIL and SMAD7 genes 2 to 4-fold, whereas inhibition of TGF-β signaling with the small molecule inhibitor SB-431542 reduced target mRNA expression by 50–90% (Fig. 2A). SMAD6 expression remained unaffected. The well-known BMP target genes ID1, ID2 and ID3 displayed a 50–60% reduction in expression on TGF-β stimulation, while TGF-β inhibition stimulated expression of these genes to approximately 3-fold. BMP stimulation induced gene expression of SKIL, SMAD7, SMAD6, ID1, ID2 and ID3 between 2- and 5-fold, while SERPINE1 and PMEPA1 were unaffected (Fig. 2B). Based on the response to TGF-β and BMP stimulation, we categorized the genes into four groups (Table 1). The first group is TGF-β responsive and consists of genes SERPINE1 and PMEPA1. The second group is both TGF-β and BMP responsive and consists of SKIL and SMAD7. SMAD6 belongs to the third group and is BMP responsive. And the fourth group consists of genes that become activated by BMP, but are repressed upon TGF-β stimulation. Expression of members of the MLL3 or MLL4 methyltransferase complexes is not affected by stimulation or repression of either TGF-β or BMP (Fig. S1A and S1B).
PTIP and PA1 knockdown reduces TGF-β specific target gene expression – To investigate the interaction between TGF-β signaling with PTIP and PA1 in the context of MLL3 and MLL4 protein complexes, siRNA-mediated knockdown efficiencies of PTIP and PA1 expression were analyzed in U-2 OS (Fig. 3). Reduction in expression was tested using qRT-PCR for all genes and immunoblot analysis. mRNA expression analysis showed knockdown efficiencies of 70–95% for PA1, PTIP and SMAD4 (Fig. 3A). For MLL3 and MLL4 a single round of knockdown yielded mRNA knockdown efficiencies of approximately 50% (data not shown). To obtain a better knockdown, cells were transfected twice to obtain a knockdown efficiency of 70–90% (Fig. 3B). Immunoblotting confirmed the expression analysis and, in addition, this showed a strong reduction of H3K4me3 levels with ASH2L knockdown (Fig 3C). This reduction is in line with results from our previous studies where ASH2L but not the enzymatically redundant MLL3 and MLL4 were identified as regulators of global H3K4 methylation levels in an unbiased siRNA screen.29

Next, expression levels of the previously-tested TGF-β and BMP target genes were investigated. SERPINE1 and PMEPA1 gene expression was reduced upon PA1, PTIP and the combination of PA1 and PTIP knockdown, strongly suggesting a functional link (Fig. 4A). Interestingly, the BMP stimulated genes ID1, ID2 and ID3 (Fig. 2B) were not affected after PA1 or PTIP knockdown (Fig. 4A), suggesting a specific link between TGF-β signaling and PA1 and PTIP to this subgroup of genes, but not to the BMP stimulated genes. SMAD6 mRNA expression is induced after PA1 knockdown. However, this does not seem to be linked
to PTIP, because knockdown of PTIP does not affect expression of SMAD6. PA1 and PTIP are present in both the MLL3 and MLL4 complexes, but also as an independent hetero-dimeric complex. To investigate involvement of the MLL3 and/or MLL4 histone methyltransferase complexes, we determined the effects after MLL3 or MLL4 siRNA knockdown. Interestingly, knockdown of MLL4 but not MLL3 resulted in reduced expression of the SERPINE1 and PMEPA1 genes, suggesting that PA1 and PTIP mediate TGF-β SMAD transcription as part of the MLL4 complex (Fig. 4B). Knockdown of MLL4 resulted in induced expression of BMP induced genes SMAD6, ID1, ID2 and ID3.

Overall, these data indicate that TGF-β mediated transcription activation involves the PA1 and PTIP subunits of the histone methyltransferase complex MLL4.

Table 1. Genes and TGF-β and BMP responses.

| GENE   | TGF-β | BMP | GROUP               |
|--------|-------|-----|---------------------|
| SERPINE1 | ↑     | –   | TGF-β responsive    |
| PMEPA1  | ↑     | –   | TGF-β responsive    |
| SKIL    | ↑     | ↑   | TGF-β and BMP responsive |
| SMAD7   | ↑     | ↑   | TGF-β and BMP responsive |
| SMAD6   | –     | ↑   | BMP responsive      |
| ID1     | ↓     | ↑   | BMP responsive, TGF-β repressed |
| ID2     | ↓     | ↑   | BMP responsive, TGF-β repressed |
| ID3     | ↓     | ↑   | BMP responsive, TGF-β repressed |

**Discussion**

SMAD-mediated signaling results in the altered mRNA expression of selected gene sets. Although TGF-β and BMP signaling has been studied extensively, relatively little is known about the downstream coactivator complexes that cooperate with SMAD signaling to facilitate target gene transcription. Both TGF-β and BMP SMAD signaling complexes have been shown to recruit the co-activating proteins CREB-binding protein (CBP), p300 and the PCAF subunits of the SAGA and ATAC histone acetyltransferases to promote target gene transcription.\(^6\)\(^-\)\(^8\) We show that TGF-β- but not BMP-activated SMADs interact with the MLL4 methyltransferase complex suggesting a TGF-β-specific target gene response pathway. The SMAD2, 3 and 4 proteins interact directly with the PA1 and PTIP proteins in transient transfection experiments. Knockdown of PA1, PTIP and MLL4 result in reduced expression of the TGF-β responsive genes SERPINE1 and PMEPA1.

MLL3 and MLL4 methyltransferase complexes are required for methylation of H3K4 and for demethylation of H3K27 at enhancer elements in the genome.\(^16\)\(^,\)\(^17\) Recently, MLL4 was described to be essential for cell fate transition by recruiting p300 to the enhancer sites, independently of its methyltransferase activity.\(^30\) Furthermore, motif analysis showed SMAD2/3 binding sites to be
enriched at MLL4 occupied active enhancers. Additionally, UCSC genome browser track analysis for SMAD3 and MLL4 show co-localization for the TGF-β responsive genes SERPINE1 and PMEPA1 (Fig. S2).

Both SMADs and MLL methyltransferase genes are often found mutated in various forms of cancers. The SMAD2 and SMAD4 gene have mutations in 5.7% and 9.8%, respectively in colon and rectal carcinomas. MLL4 is most often (but not exclusively) found mutated in bladder urothelial carcinoma and uterine corpus cell carcinomas (7.1% and 8.3%, respectively). Furthermore, MLL4 is mutated in 2.1% of all colorectal cancers. Enhancer profiling in colon cancers has shown thousands of sites that are either gained or lost H3K4me1 levels compared to healthy tissues. The link between SMAD-mediated TGF-β signaling and the MLL4 methyltransferase allows for new opportunities to study and possibly identify novel treatment options for these types of cancer and, thus, the findings reported warrant further research.

Figure 3. Knockdown efficiency of siRNA treated U-2 OS. (A-C) Cells were reverse transfected with siNT, siPA1, siPTIP, siPA1 + siPTIP or siSMAD4 or with siNT, siASH2L, siMLL3 or siMLL4 and treated again after 24 h and collected after 72 hours. (A-B) Samples were analyzed using qRT-PCR. Bars represent average expression corrected for ACTIN and normalized to the siNT control. Error bars represent standard deviation. Technical replication n = 3 for all samples. (C) Samples were analyzed on immunoblot using PA1, SMAD4, ASH2L, H3K4me3, H3 and Tubulin antibodies. * indicates nonspecific antibody background binding.

Figure 4. TGF-β target genes are selectively affected by PA1 and PTIP knockdown. (A-B) Gene expression analysis of TGF-β and BMP target genes and SET/MLL complex member genes. (A) U-2 OS cells were reverse transfected with siNT, siPA1, siPTIP, siPA1 + siPTIP or siSMAD4. (B) Cells were reverse transfected with siNT, siASH2L, siMLL3 or siMLL4 and treated again after 24 h to enhance knockdown efficiency. (A-B) Samples were analyzed using qRT-PCR. Bars represent average expression corrected for ACTIN and normalized to the siNT control. Error bars represent standard deviation. Technical replication n = 3 for all samples.
Materials and methods

Plasmids

Gateway compatible pENTR-SMAD vectors$^{24,33}$ were recombined with pMT2SM-HA in an LR reaction according to manufacturer’s instructions (Thermo Fischer Scientific). GFP-PTIP and GFP-PA1 expression vectors were described before.$^{18}$

Cell culture

Cells were grown in Dulbecco’s Modified Eagle’s Medium (DMEM) containing 1 g/L or 4.5 g/L glucose for U-2 OS or HEK 293T respectively supplemented with 10% v/v fetal bovine serum, 10 mM L-glutamine, 100 U/mL penicillin and 100 U/mL streptomycin.

Stimulation and inhibition of TGF-β or BMP signaling

U-2 OS cells were counted and seeded 150,000 cells per well in a 6-well plate. Cells were left overnight to attach. Cells were treated with 10 μM SB431542 (#616461, Calbiochem) or 100 nM LDN193189 (SML0559, Sigma- Aldrich) to inhibit TGF-β or BMP signalling, respectively. After 24 h the cells were stimulated with 10 ng/mL TGF-β1 (#8915LC, Cell Signalling) or 10 ng/mL rhBMP-2 (355-BEC, R&D systems) for 6 h and collected for mRNA expression analysis.

siRNA transfection

U-2 OS cells were transfected using a reverse transfection protocol with Hiperfect (Qiagen) transfection reagent according to the manufacturer’s instructions. In short: 125 μL of a SMARTpool of four siRNAs (25 nM each) was dispensed in a 6-well plate (657165, Greiner bio-one). Transfection mix (10 μL Hiperfect in total volume of 375 μL Opti-MEM [Invitrogen] per well) was added, followed by 30 minutes incubation at room temperature. After this 70,000 U-2 OS cells/well were added in 2 mL. Cells treated with siRNA against genes listed in Fig. 3B were treated again 24 h after cell seeding. Cells were collected 72 h after the first transfection.

Transient transfection, whole cell extracts and immunoprecipitation

HEK 293T cells were counted and seeded at 150,000 cells per well in a 6-wells plate. Cells were left overnight to attach and expression vectors were transfected using polyethyleneimine (PEI). Cells were washed once with PBS and cell extracts were collected in whole cell extract buffer (50 mM Tris-HCl pH 8.0, 10% glycerol, 100 mM NaCl, 10 mM MgCl2, 0.5 mM DTT, 0.1% NP-40 and 1x protease inhibitor [Complete Protease Inhibitor Cocktail, Roche]). GFP affinity purification was essentially performed as described up to sample elution.$^{34}$

Immunoblot analysis

Cells were lysed in sample buffer (160 mM Tris-HCl pH 6.8, 4% SDS, 20% glycerol, 0.05% bromophenol blue) and equal amounts of protein were separated using 10% or 15% SDS-PAGE gels and transferred onto PVDF membrane. The membrane was developed with the appropriate antibodies and ECL and scanned using ChemiDoc Touch Imaging System (Bio-Rad).

Antibodies

Antibodies were used from the following sources: ASH2L (kind gift from Winship Herr), GAPDH (clone 6C5, mAb374, Millipore), GFP (kind gift from Geert

Table 2. Primers used for qRT-PCR.

| Primer | Sequence                  |
|--------|---------------------------|
| ACTIN Fw | AGAAAATCTGGCACCACACC    |
| ACTIN Rv | AGAGCGCTACAGGGATAGCA    |
| PA1 Fw   | CCAAGACGGCAAGAGGAGAGA   |
| PA1 Rv   | GTGGGCAATGGTGTTTTC      |
| PTIP Fw  | CATCGACCGCCAGTTATCCA   |
| PTIP Rv  | GAGGTTCTCCACCTCCTGGA   |
| ASH2L Fw | TGCTGTCATCTAGTAGGGAGATT |
| ASH2L Rv | TGGGCTCTCTGCTTCTTC     |
| MLL3 Fw  | CCCAGGAAAACAAAGAGGAGACAG |
| MLL3 Rv  | TGGGCTTACATCCATTACAAAGAT |
| MLL4 Fw  | GCAGCAGCTAGCTCCTTCCC   |
| MLL4 Rv  | CCTTGCCACATGGCTTGGG    |
| MAM4 Fw  | GCAATGGAACACAAATACTCG  |
| MAM4 Rv  | CTGCCACAAGTAAAACAACTGTC |
| KDM6A Fw | CCAGGTGAGATCAGTAGGAGAGC |
| KDM6A Rv | CTTTTGAGATCAGTAGGAGAGC |
| SERPINE1 Fw | CCAGGACCTCTGAGAATTC   |
| SERPINE1 Rv | CCGGGACTAGCCAGGTTG    |
| PMEPA1 Fw | CTGGTGCAGCCTCTTCCAT   |
| PMEPA1 Rv | CCACAGGATCTCCTGAGGAGAG |
| SKIL Fw  | GAGGCTGATATGCAGAAGACAGC |
| SKIL Rv  | CGTGGCTATGGCCCTCACAG |
| SMAD7 Fw | TCAAGAGGCTTGTTGCTTGTG |
| SMAD7 Rv | TGTTGTTGAAAGATCTCATGCGG |
| ID1 Fw   | TCTTCAAGCCGACCAGATCTC |
| ID1 Rv   | GCCCTGAGATTGGTGCTGAGA  |
| ID2 Fw   | CAGGGACTTCTGAGAAGACAGC |
| ID2 Rv   | AAGCCAGCTGCCGCCCTCGA |
| ID3 Fw   | TGACACCAACCTACACAGGAT |
| ID3 Rv   | CATGAACACGGCTTTACAGGCC |
| ID3 Rv   | GAGTCCCGAGGAGAACCTCA  |
| ID3 Rv   | CTGGGGCTTGTGATGGGAAA  |
Kops), H3 (Ab1791; Abcam), H3K4me3 (pAb003-050, Diagenode), 29 HA (3F10-HRP, Roche), PA1 (kind gift from Junjie Chen), SMAD4 (#9515, Cell Signaling), α-Tubulin (DM1A, CP06, Calbiochem).

Expression analysis qRT-PCR

Cells were lysed and total RNA was isolated from cells using RNeasy kit (Qiagen) including a DNase treatment using RNase-Free DNase (Qiagen) according to manufacturer’s instructions. 500 ng of total RNA was used for cDNA synthesis (Superscript II, Invitrogen) using random primers. A 25 μL qRT-PCR reaction was performed in a CFX Connect Real-Time PCR Detection System (Bio-Rad) using iQ SYBR Green Supermix (Bio-Rad) Primers used for qRT-PCR can be found in Table 2.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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Author contributions

RB and ST performed expression analysis. HvT provided technical assistance and contributed to the immunoprecipitation experiments. MT coordinated the study. RB wrote the paper, which was corrected by MT and reviewed by all authors.

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