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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 confirm TGF-β-specific genes to be regulated by the MLL4 complex, which links TGF-β signaling to transcription regulation by the MLL4 methyltransferase complex.

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).[1–3] 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[4,5] Furthermore, SMAD complexes bind to the coactivator complex p300 and the PCAF histone acetyltransferases.[6–8] Recruitment of p300 or the PCAF subunit of SAGA and ATAC complexes[9] 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.[10–13]

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.[14] H3K4 mono-methylation (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[15] while Mixed Lineage Leukemia 3 (MLL3, also named lysine methyl transferase 2C (KMT2C)) and MLL4 (KMT2D)

complexes are maintaining H3K4 mono-methylation at enhancer sites.[16,17]

The SET1/MLL complexes all contain the WDR5, RBBP5, ASH2L and DPY30 proteins that make up the common WRAD core.[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).[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.[18,21,22] SMAD2 was previously identified as an interaction partner of PTIP.[18,23] However, the link to the MLL3 and MLL4 methyltransferases 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[23] and was confirmed previously by us using an unbiased proteomics approach.[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.[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.[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.[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).
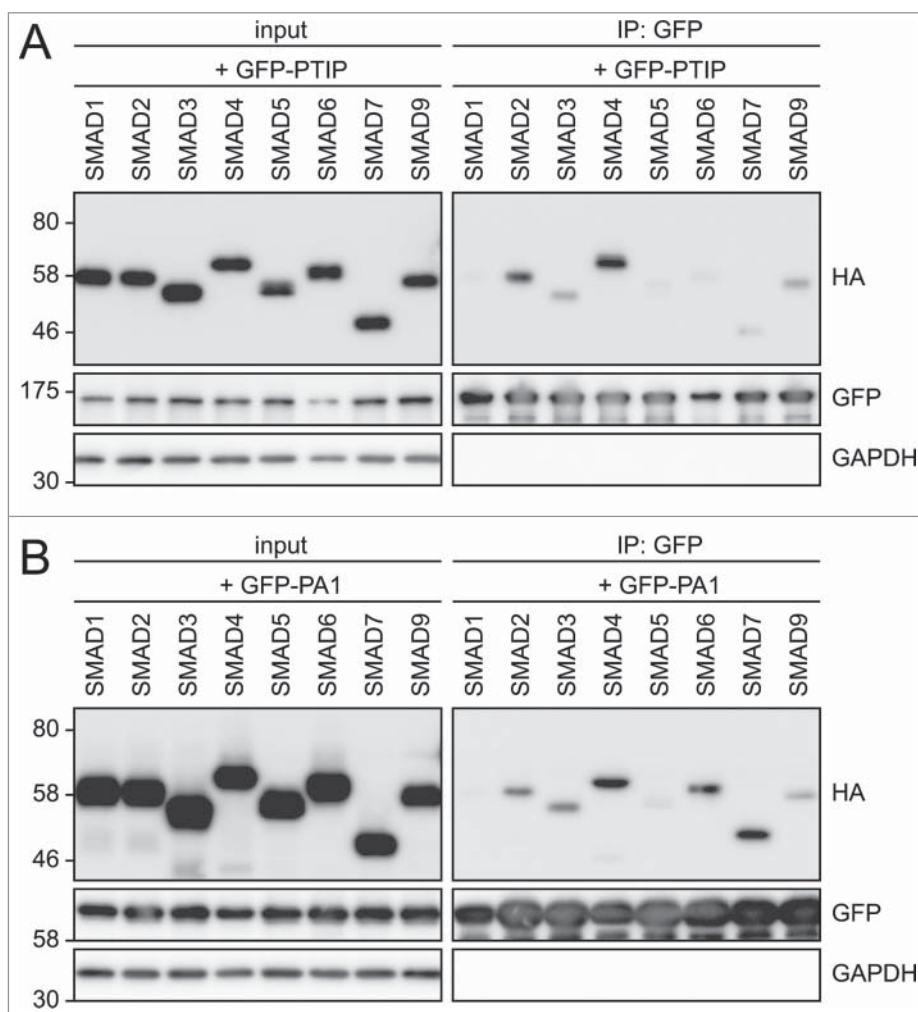
Figure 1. TGF-β activated R-SMADs interact with PTIP and PA1. (A-B) HEK 293T cells were co-transfected with HA-tagged SMADs and GFP-tagged PA1 or PTIP expression plasmids. Affinity purification of GFP fusions was performed on whole cell extracts. Samples were analyzed by immunoblotting using HA, GFP and GAPDH antibodies. (A) Co-immunoprecipitation for GFP-PTIP. (B) Co-immunoprecipitation for GFP-PA1. For input control 1.5% of the extract was analyzed.

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
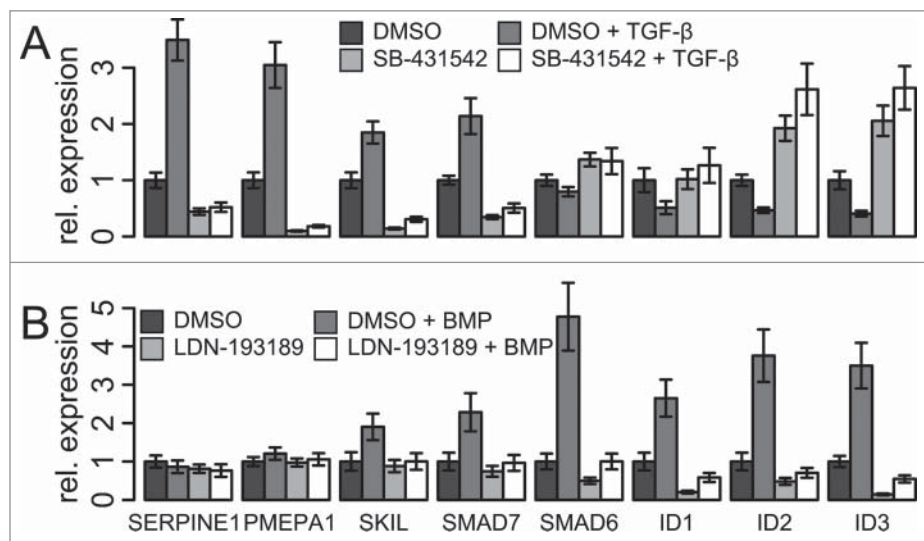
Figure 2. Investigation of TGF-β and BMP target genes in U-2 OS cells. (A-B) Gene expression analysis of TGF-β and BMP target genes. (A) U-2 OS cells were treated with 10 μM SB-431542 inhibitor for 24 h and subsequently stimulated with 10 ng/ml TGF-β for 6 h as indicated. (B) U-2 OS cells were treated with 100 nM LDN-193189 inhibitor for 24 h and subsequently stimulated with 10 ng/ml BMP for 6 h as indicated. (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.

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-B	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
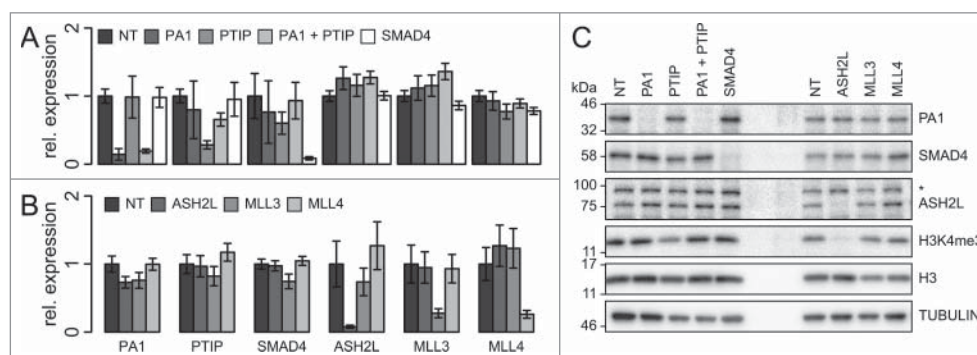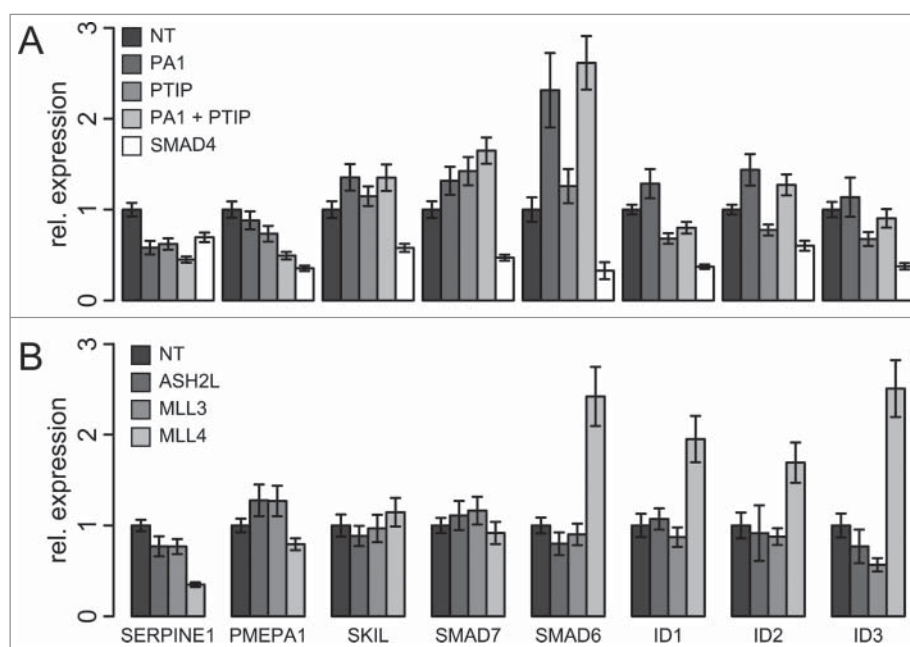
Figure 3. Knockdown efficiency of siRNA treated U-2 OS. (A-C) Cells were reverse transfected with siNT, siPA1, siPTIP, siPA1 + siPTIP or siSMAD4 or with siNT, siASH2L, siMLL3 or siMLL4 and treated again after 24 h and collected after 72 hours. (A-B) Samples were analyzed using qRT-PCR. Bars represent 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.

enriched at MLL4 occupied active enhancers.[30] 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.[31] 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.[32] 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 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 Signaling) 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 MgCl$_2$, 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	AGAGGCGTACAGGGATAGCA
PA1 Fw	CCCAGAGCGAAGAGGAGAGA
PA1 Rv	GTGGGCATGTGTGGTTTTTCC
PTIP Fw	CATCGACCCGCAGGTTATTCA
PTIP Rv	GAGCTTCTCCCACCTCTGGA
ASH2L Fw	TGCTGTGTCTACTAGTGGGAATTT
ASH2L Rv	TTGGCTCCTCGTCCTTTTC
MLL3 Fw	CCACGAAAACAAAGAGGACAG
MLL3 Rv	TGGGTGCTTACACTTACACAAGAT
MLL4 Fw	GTCTTCACTGACGCCTCTCC
MLL4 Rv	GCAGCATGTCAGCCTCTTTT
SMAD4 Fw	CCTGTTCACAATGAGCTTGC
SMAD4 Rv	GCAATGGAACACCAATACTCAG
KDM6A Fw	CTGCACAAGTAAAAGCAACTGTC
KDM6A Rv	CTTTTGGAGATACTGAATAGCATAGC
SERPINE1 Fw	AAGGCACCTCTGAGAACTTCA
SERPINE1 Rv	CCCAGGACTAGGCAGGTG
PMEPA1 Fw	CTGTCTGCACGGTCCTTCAT
PMEPA1 Rv	CCACAGGCATCCTTCTGAGG
SKIL Fw	GAGGCTGAATATGCAGGACAG
SKIL Rv	CTTGCCTATCGGCCTCAG
SMAD7 Fw	TCAAGAGGCTGTGTTGCTGTG
SMAD7 Rv	TGGTTTGAGAAAATCCATCGGGT
SMAD6 Fw	TTCTCAGACGCCAGCATGTC
SMAD6 Rv	GCCCTGAGGTAGGTCGTAGA
ID1 Fw	CAGGGACCTTCAGTTGGAGC
ID1 Rv	AACGCATGCCGCCTCG
ID2 Fw	TGACCACCCTCAACACGGAT
ID2 Rv	CATGAACACCGCTTATTCAGCC
ID3 Fw	GAGTCCCGAGAGGCACTCA
ID3 Rv	CTCGGCTGTCTGGATGGGAA

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.

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.

References

1. Lechleider RJ, de Caestecker MP, Dehejia A, Polymeropoulos MH, Roberts AB. Serine phosphorylation, chromosomal localization, and transforming growth factor-beta signal transduction by human bsp-1. J Biol Chem. 1996;271:17617–20. doi:10.1074/jbc.271.30.17617. PMID:8663601.

2. Yingling JM, Das P, Savage C, Zhang M, Padgett RW, Wang XF. Mammalian dwarfins are phosphorylated in response to transforming growth factor beta and are implicated in control of cell growth. Proc Natl Acad Sci U S A. 1996;93:8940–4. doi:10.1073/pnas.93.17.8940. PMID:8799132.

3. Zhang Y, Feng X, We R, Derynck R. Receptor-associated Mad homologues synergize as effectors of the TGF-beta response. Nature. 1996;383:168–72. doi:10.1038/383168a0. PMID:8774881.

4. Varelas X, Sakuma R, Samavarchi-Tehrani P, Peerani R, Rao BM, Dembowy J, Yaffe MB, Zandstra PW, Wrana JL. TAZ controls Smad nucleocytoplasmic shuttling and regulates human embryonic stem-cell self-renewal. Nat Cell Biol. 2008;10:837–48. doi:10.1038/ncb1748. PMID:18568018.

5. Kawarada Y, Inoue Y, Kawasaki F, Fukuura K, Sato K, Tanaka T, Itoh Y, Hayashi H. TGF-beta induces p53/Smads complex formation in the PAI-1 promoter to activate transcription. Sci Rep. 2016;6:35483. doi:10.1038/srep35483. PMID:27759037.

6. Janknecht R, Wells NJ, Hunter T. TGF-beta-stimulated cooperation of smad proteins with the coactivators CBP/p300. Genes Dev. 1998;12:2114–9. doi:10.1101/gad.12.14.2114. PMID:9679056.

7. Pearson KL, Hunter T, Janknecht R. Activation of Smad1-mediated transcription by p300/CBP. Biochim Biophys Acta. 1999;1489:354–64. doi:10.1016/S0167-4781(99)00166-9. PMID:10673036.

8. Pouponnot C, Jayaraman L, Massague J. Physical and functional interaction of SMADs and p300/CBP. J Biol Chem. 1998;273:22865–8. doi:10.1074/jbc.273.36.22865. PMID:9722503.

9. Spedale G, Timmers HT, Pijnappel WW. ATAC-king the complexity of SAGA during evolution. Genes Dev. 2012;26:527–41. doi:10.1101/gad.184705.111. PMID:22426530.

10. Dhalluin C, Carlson JE, Zeng L, He C, Aggarwal AK, Zhou MM. Structure and ligand of a histone acetyltransferase bromodomain. Nature. 1999;399:491–6. doi:10.1038/20974. PMID:10365964.

11. Jacobson RH, Ladurner AG, King DS, Tjian R. Structure and function of a human TAFII250 double bromodomain module. Science. 2000;288:1422–5. doi:10.1126/science.288.5470.1422. PMID:10827952.

12. Lange M, Kaynak B, Forster UB, Tonjes M, Fischer JJ, Grimm C, Schlesinger J, Just S, Dunkel I, Krueger T, et al. Regulation of muscle development by DPF3, a novel histone acetylation and methylation reader of the BAF chromatin remodeling complex. Genes Dev. 2008;22:2370–84. doi:10.1101/gad.471408. PMID:18765789.

13. Sanchez R, Zhou MM. The role of human bromodomains in chromatin biology and gene transcription. Curr Opin Drug Discov Devel. 2009;12:659–65. PMID:19736624.

14. Vermeulen M, Mulder KW, Denissov S, Pijnappel WW, van Schaik FM, Varier RA, Baltissen MP, Stunnenberg HG, Mann M, Timmers HT. Selective anchoring of TFIID to nucleosomes by trimethylation of histone H3 lysine 4. Cell. 2007;131:58–69. doi:10.1016/j.cell.2007.08.016. PMID:17884155.

15. Wu M, Wang PF, Lee JS, Martin-Brown S, Florens L, Washburn M, Shilatifard A. Molecular regulation of H3K4 trimethylation by Wdr82, a component of human Set1/

COMPASS. Mol Cell Biol. 2008;28:7337–44. doi:10.1128/MCB.00976-08. PMID:18838538.

16. Herz HM, Mohan M, Garruss AS, Liang K, Takahashi YH, Mickey K, Voets O, Verrijzer CP, Shilatifard A. Enhancer-associated H3K4 monomethylation by Trithorax-related, the Drosophila homolog of mammalian Mll3/Mll4. Genes Dev. 2012;26:2604–20. doi:10.1101/gad.201327.112. PMID:23166019.

17. Hu D, Gao X, Morgan MA, Herz HM, Smith ER, Shilatifard A. The MLL3/MLL4 branches of the COMPASS family function as major histone H3K4 monomethylases at enhancers. Mol Cell Biol. 2013;33:4745–54. doi:10.1128/MCB.01181-13. PMID:24081332.

18. van Nuland R, Smits AH, Pallaki P, Jansen PW, Vermeulen M, Timmers HT. Quantitative dissection and stoichiometry determination of the human SET1/MLL histone methyltransferase complexes. Mol Cell Biol. 2013;33:2067–77. doi:10.1128/MCB.01742-12. PMID:23508102.

19. Cho YW, Hong T, Hong S, Guo H, Yu H, Kim D, Guszczynski T, Dressler GR, Copeland TD, Kalkum M, et al. PTIP associates with MLL3- and MLL4-containing histone H3 lysine 4 methyltransferase complex. J Biol Chem. 2007;282:20395–406. doi:10.1074/jbc.M701574200. PMID:17500065.

20. Patel SR, Kim D, Levitan I, Dressler GR. The BRCT-domain containing protein PTIP links PAX2 to a histone H3, lysine 4 methyltransferase complex. Dev Cell. 2007;13:580–92. doi:10.1016/j.devcel.2007.09.004. PMID:17925232.

21. Starnes LM, Su D, Pikkupeura LM, Weinert BT, Santos MA, Mund A, Soria R, Cho YW, Pozdnyakova I, Kubec Højfeldt M, et al. A PTIP-PA1 subcomplex promotes transcription for IgH class switching independently from the associated MLL3/MLL4 methyltransferase complex. Genes Dev. 2016;30:149–63. PMID:26744420.

22. Gong Z, Cho YW, Kim JE, Ge K, Chen J. Accumulation of Pax2 transactivation domain interaction protein (PTIP) at sites of DNA breaks via RNF8-dependent pathway is required for cell survival after DNA damage. J Biol Chem. 2009;284:7284–93. doi:10.1074/jbc.M809158200. PMID:19124460.

23. Shimizu K, Bourillot PY, Nielsen SJ, Zorn AM, Gurdon JB. Swift is a novel BRCT domain coactivator of Smad2 in transforming growth factor beta signaling. Mol Cell Biol. 2001;21:3901–12. doi:10.1128/MCB.21.12.3901-3912.2001. PMID:11359898.

24. Baas R, Sijm A, van Teeffelen HA, van Es R, Vos HR, Timmers HT. Quantitative Proteomics of the SMAD (Suppressor of Mothers Against Decapentaplegic) Transcription Factor Family Identifies Importin 5 as a Bone Morphogenic Protein Receptor-SMAD specific Importin. J Biol Chem. 2016;291(46):24121–32. doi:10.1074/jbc.M116.748582. PMID:27703004.

25. de Jong DS, Vaes BL, Dechering KJ, Feijen A, Hendriks JM, Wehrens R, Mummery CL, van Zoelen EJ, Olijve W, Steegenga WT. Identification of novel regulators associated with early-phase osteoblast differentiation. J Bone Miner Res. 2004;19:947–58. doi:10.1359/JBMR.040216. PMID:15125793.

26. Levy L, Hill CS. Smad4 dependency defines two classes of transforming growth factor {beta} (TGF-{beta}) target genes and distinguishes TGF-{beta}-induced epithelial-mesenchymal transition from its antiproliferative and migratory responses. Mol Cell Biol. 2005;25:8108–25. doi:10.1128/MCB.25.18.8108-8125.2005. PMID:16135802.

27. Locklin RM, Riggs BL, Hicok KC, Horton HF, Byrne MC, Khosla S. Assessment of gene regulation by bone morphogenetic protein 2 in human marrow stromal cells using gene array technology. J Bone Miner Res. 2001;16:2192–204. doi:10.1359/jbmr.2001.16.12.2192. PMID:11760832.

28. Prashar P, Yadav PS, Samarjeet F, Bandyopadhyay A. Microarray meta-analysis identifies evolutionarily conserved BMP signaling targets in developing long bones. Dev Biol. 2014;389:192–207. doi:10.1016/j.ydbio.2014.02.015. PMID:24583261.

29. Baas R, Lelieveld D, van Teeffelen H, Lijnzaad P, Castelijns B, van Schaik FM, Vermeulen M, Egan DA, Timmers HT, de Graaf P. A novel microscopy-based high-throughput screening method to identify proteins that regulate global histone modification levels. J Biomol Screen. 2014;19:287–96. doi:10.1177/1087057113515024. PMID:24334265.

30. Wang C, Lee JE, Lai B, Macfarlan TS, Xu S, Zhuang L, Liu C, Peng W, Ge K. Enhancer priming by H3K4 methyltransferase MLL4 controls cell fate transition. Proc Natl Acad Sci U S A. 2016;113:11871–6. doi:10.1073/pnas.1606857113. PMID:27698142.

31. Kandoth C, McLellan MD, Vandin F, Ye K, Niu B, Lu C, Xie M, Zhang Q, McMichael JF, Wyczalkowski MA, et al. Mutational landscape and significance across 12 major cancer types. Nature. 2013;502:333–9. doi:10.1038/nature12634. PMID:24132290.

32. Akhtar-Zaidi B, Cowper-Sal-lari R, Corradin O, Saiakhova A, Bartels CF, Balasubramanian D, Myeroff L, Lutterbaugh J, Jarrar A, Kalady MF, et al. Epigenomic enhancer profiling defines a signature of colon cancer. Science. 2012;336:736–9. doi:10.1126/science.1217277. PMID:22499810.

33. Hartley JL, Temple GF, Brasch MA. DNA cloning using in vitro site-specific recombination. Genome Res. 2000;10:1788–95. doi:10.1101/gr.143000. PMID:11076863.

34. Baymaz HI, Spruijt CG, Vermeulen M. Identifying nuclear protein-protein interactions using GFP affinity purification and SILAC-based quantitative mass spectrometry. Methods Mol Biol. 2014;1188:207–26. doi:10.1007/978-1-4939-1142-4_15. PMID:25059614.