Regulation of transforming growth factor-β and bone morphogenetic protein signaling by transcriptional co-activator GCN5

転写共役因子 GCN5 による TGF-β・BMP シグナル伝達の制御機構
Regulation of transforming growth factor-β and bone morphogenetic protein signaling by transcriptional co-activator GCN5

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The abbreviations used are: TGF-β, transforming growth factor-β; TβR-I, TGF-β type I receptor; BMP, bone morphogenetic protein; BMPR-IB, BMP type IB receptor; R-Smad, receptor-regulated Smad; Co-Smad, common-partner Smad; I-Smad, inhibitory Smad; GCN5, general control of amino acid synthesis 5; CBP, CREB binding protein; PCAF, p300 CBP associated factor; GNAT, GCN5-related N-acetyltransferase; HAT, histone acetyltransferase; HA, hemagglutinin; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; DMEM, Dulbecco’s modified Eagle’s medium; FBS, fetal bovine serum; DNA-P, DNA affinity purification; ChIP, chromatin immunoprecipitation; RNAi, RNA interference.
Introduction
Members of the transforming growth factor-β (TGF-β) superfamily are multifunctional proteins that regulate various cellular responses, including cell proliferation, differentiation, migration, and apoptosis (Roberts & Sporn 1990). The TGF-β superfamily includes TGF-βs, activins and inhibins, bone morphogenetic proteins (BMPs), and Müllerian inhibiting substance. Members of the TGF-β superfamily bind to type II and I serine/threonine kinase receptors and transduce intracellular signals by Smad proteins (Heldin et al. 1997; Shi & Massagué 2003). Type II receptor kinases are constitutively active; upon ligand binding and complex formation with type I receptors, type II receptor kinases transphosphorylate type I receptors, resulting in activation of Smads by type I receptor kinases.

Smads are classified into three groups depending on their roles in signaling; receptor-regulated Smads (R-Smads), common-partner Smads (Co-Smads), and inhibitory Smads (I-Smads). R-Smads are direct substrates of the type I receptors. R-Smads are phosphorylated at the C-terminal SSXS motif by serine/threonine kinase receptors and form heteromeric complexes with Co-Smads. The Smad complexes translocate into the nucleus, where they regulate the transcription of various target genes. Smad3 and Smad4, but not Smad2, directly bind to DNA through their N-terminal MH1 domains (Yagi et al. 1999). The Smad binding element (SBE), containing a CAGACA sequence, is known as a consensus DNA sequence for Smads 3 and 4 binding, and is present in promoter regions of many TGF-β response genes, including the plasminogen activator inhibitor-1 (PAI-1) gene (Dennler et al. 1998).

Transcriptional co-activators p300 and CREB binding protein (CBP) have been shown to interact with R-Smads in a ligand-dependent manner (Feng et al. 1998; Janknecht et al. 1998; Nishihara et al. 1998; Shen et al. 1998). p300 and CBP have intrinsic histone acetyltransferase (HAT) activity, which facilitates transcription by decreasing chromosome condensation through histone acetylation and by increasing the accessibility of transcription factors with the basal transcription machinery (Bannister & Kouzarides 1996). Thus, p300/CBP positively regulate Smad-mediated transcriptional activation. In addition to p300/CBP, another transcriptional co-activator, p300 CBP associated factor (PCAF), which belongs to the GCN5-related N-acetyltransferase (GNAT) superfamily, has been reported to interact with Smads 2 and 3, to facilitate transcription induced by TGF-β (Itoh et al. 2000). Other proteins involved in Smad regulation include c-Ski, SnoN and TGIF. These proteins interact with Smads 2, 3 and/or 4 in the nucleus. They compete with p300/CBP and recruit histone deacetylases to Smad complexes, resulting in transcriptional repression (Akiyoshi et al. 1999; Stroschein et al. 1999; Wotton et al. 1999).

GCN5 is a transcriptional co-activator that functions as a HAT to promote transcriptional activation of various genes (Candau et al. 1996; Wang et al. 1997). GCN5 is structurally related to PCAF; therefore, it is also termed PCAF-B (Yamauchi et al. 2000). However, the function of GCN5 in TGF-β superfamily signaling was previously unknown. In the present study, we identified GCN5 as a Smad-binding transcriptional co-activator. In contrast to PCAF, which preferentially enhances transcriptional activity induced by TGF-β, GCN5...
enhances transcriptional activities for both TGF-β and BMP.

Results

GCN5 interacts with activated Smad complexes—To identify new components of transcriptional complexes containing Smad proteins, we performed DNA affinity purification (DNAP) using human breast cancer MCF-7 cell nuclear extract. Biotinylated 3xCAGA oligonucleotide, to which activated Smads 3 and 4 binds directly (Dennler et al. 1998), was used to purify DNA-binding proteins. The CAGA-binding proteins from the nuclear extract were separated by SDS-PAGE followed by silver nitrate staining (Fig. 1A). The proteins that bound to wild-type 3xCAGA, but not to mutated 3xCAGA, were subjected to in-gel trypsin digestion, followed by mass spectrometry analysis using Autoflex (Bruker). Peptide mass fingerprinting, and database searching with the peptide mass spectra obtained, revealed that one of the proteins was human GCN5 (Fig. 1A, arrow).

To further characterize the association of GCN5 with the CAGA sequence, we performed the DNAP assay using lysates of transfected COS7 cells (Fig. 1B). GCN5 bound to CAGA in the presence of both Smad3 and a constitutively active form of TGF-β type I receptor, TβR-I(TD), whereas GCN5 alone did not bind to CAGA. Moreover, GCN5 was not detected when mutant 3xCAGA was used. These findings suggest that GCN5 is incorporated in the activated Smad complexes. It is important to note that the MCF-7 nuclear extract used for purification in the present study was derived from cells that were not stimulated with TGF-β. We therefore tested whether the extract contained activated Smad complexes by immunoblotting. Phospho-Smad2, phospho-Smad3, and Smad4 were detected in the nuclear extract (data not shown), suggesting that MCF-7 is activated by endogenous TGF-β to some extent, and that the nuclear extract contains activated Smad complexes.

To investigate whether GCN5 is indeed incorporated in the activated Smad complex on DNA in vivo, we performed a chromatin immunoprecipitation (ChIP) assay using the promoter of a TGF-β-responsive endogenous target gene, human PAI-1 (Fig. 2A). Since an anti-GCN5 antibody that could be used for immunoprecipitation experiments was not available, we generated a stable HaCaT human keratinocyte cell line expressing FLAG-GCN5. Expression of FLAG-GCN5 in the selected clone (HG15) was detected by immunoblotting analysis (Fig. 2B). One hour after treatment with TGF-β, binding of endogenous Smad2/3 to one of the CAGA regions in the PAI-1 promoter (nucleotides −280−272) was observed (Fig. 2C, left panel). Moreover, immunoprecipitation of FLAG-GCN5 revealed that the recruitment of FLAG-GCN5 was greatly increased when Smad2/3 binding occurred (Fig. 2C, right panel). The binding of FLAG-GCN5 in the absence of TGF-β was at background levels for protein G (data not shown). The recruitment of Smad2/3 and GCN5 was also detected in other CAGA regions in the human PAI-1 promoter (nucleotides −730−722 and nucleotides −580−572, data not shown). These data suggested that GCN5 is recruited to the promoter region of the PAI-1 gene together with Smad2/3 in a TGF-β-dependent manner.

GCN5 associates with R-Smads for TGF-β and BMP in a ligand-dependent manner.
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We next determined the physical interaction of GCN5 with all R-Smads and with Co-Smad in transfected COS7 cells. GCN5 associated with Smads 2 and 3 in the presence of TβR-I(TD), and with Smads 1 and 5 (but not Smad4 or Smad8) in the presence of constitutively active BMP type IB receptor (BMPR-IB(QD)) (Fig. 3A).

PCAF, which is structurally similar to GCN5, also potentiates TGF-β signaling (Itoh et al. 2000). To determine the differences between GCN5 and PCAF, we examined physical interactions of PCAF with Smads using transfected COS7 cells (Fig. 3B). PCAF associated with Smad2 and Smad3 in a ligand-dependent fashion, as previously reported (Itoh et al. 2000), whereas it failed to associate with Smad1, Smad5, Smad8, and Smad4. From observations of the differences in association with Smad1 and Smad5, it was thought that GCN5 and PCAF might play different roles in BMP signaling.

We next determined the GCN5-interacting domain of R-Smads using deletion mutants of Smad3 (Fig. 3C). GCN5 binds to Smad3C (MH2 domain + Linker region) and Smad3D (MH2 domain), but not to Smad3A (MH1 domain) or Smad3B (MH1 domain + Linker region), suggesting that GCN5 associates with R-Smads through their MH2 domains.

To determine the Smad-interaction region in GCN5, we prepared two deletion mutants of GCN5, i.e. GCN5N and GCN5C (Fig. 3D, bottom). GCN5N lacks the HAT and bromo domains in the C-terminal region, whereas GCN5C possesses them, but lacks the N-terminal region. We expressed full-length GCN5, or either of its two deletion mutants, together with Smad3 and TβR-I(TD) in COS7 cells, and determined their interaction (Fig. 3D, top). Both GCN5N and GCN5C interacted with Smad3 in the presence of TβR-I(TD), suggesting that GCN5 binds to Smad3 through at least two regions in GCN5.

GCN5 enhances TGF-β and BMP signaling—Smads have intrinsic transcriptional activity when fused to the Gal4 DNA-binding domain (Liu et al. 1996; Liu et al. 1997). In order to determine whether GCN5 potentiates the intrinsic transcriptional activity of TGF-β, R mutant mink lung epithelial (Mv1Lu) cells lacking TβR-I were transfected with a luciferase reporter gene containing multiple Gal4 binding sites upstream of a minimal promoter, Gal4-Smad3 constructs, TβR-I(TD), and GCN5 (Fig. 4A). Gal4-Smad3 facilitated TGF-β dependent transcription, which was further enhanced in the presence of GCN5. Furthermore, we examined the transcriptional activity of GCN5 in TGF-β signaling using (CAGA)₉-MLP-lux in three different types of cells. Transcriptional activation of (CAGA)₉-MLP-lux by TGF-β was enhanced by expression of GCN5 in C2C12 cells (Fig. 4B), 293T cells (Fig. 4C), and R mutant Mv1Lu cells (data not shown). These results demonstrate that GCN5 positively regulates TGF-β signaling.

We next investigated the effect of the deletion mutant GCN5N on the transcriptional activity induced by TGF-β. GCN5N lacks a catalytic domain, but contains a Smad-interacting region. Transfection of GCN5N suppressed transcriptional activation of (CAGA)₉-MLP-lux induced by TβR-I(TD) in a dominant-negative fashion (Fig. 4D).

Because GCN5, but not PCAF, associates with BMP-specific R-Smads (see
Fig. 3A and B), we next tested the effects of GCN5 and PCAF on BMP signaling with a BMP-responsive reporter assay using Id1-MLP-Lux. As shown in Fig. 4E, while GCN5 potentiated the transcriptional activation induced by BMPR-IB(QD), PCAF had no effect. These findings suggest that GCN5 is involved in BMP signaling as well as in TGF-β signaling, whereas PCAF is likely to play a less important role in BMP signaling.

*Endogenous GCN5 is required for TGF-β signaling*—To confirm the function of GCN5 in TGF-β signaling, we employed RNA interference (RNAi) to inhibit endogenous expression of GCN5. The efficiency of the specific RNAi oligonucleotide for GCN5 was confirmed by the reduction in mRNA for endogenous GCN5 (Fig. 5A) and expression of co-transfected FLAG-GCN5 protein (Fig. 5B) in 293T cells. The TGF-β-induced transcriptional activity was significantly reduced in the presence of RNAi specific for GCN5 in transfected 293T cells (Fig. 5C). Similar results were obtained by using HaCaT cells (data not shown). These data support the conclusion that GCN5 functions as a co-activator in the Smad signaling pathway.

**Discussion**

In the present study, we identified GCN5 as a Smad-binding transcriptional co-activator. GCN5 is structurally similar to PCAF which was previously identified as a co-activator for TGF-β-specific R-Smads (Itoh et al. 2000). Similar to PCAF, GCN5 binds to TGF-β-specific R-Smads in a ligand-dependent manner in transfected COS7 cells. The binding region in Smad3 is its MH2 domain, which has an intrinsic transcriptional activity. Many transcriptional co-activators, such as p300/ CBP, PCAF and ARCI05, have been reported to interact with Smads via MH2 domain (Feng et al. 1998; Janknecht et al. 1998; Nishihara et al. 1998; Shen et al. 1998; Itoh et al. 2000; Kato et al. 2002). However, it is currently unknown whether these transcriptional co-activators play similar or distinct roles under physiological conditions. It should also be determined in the future whether multiple transcriptional co-activators interact with Smads at the same time, and regulate transcription of target genes in cooperative fashions. Thus, a further fine mapping of the GCN5-binding in Smads should be carried out in the future.

Human GCN5 consists of 837 amino acid residues, with the HAT domain located at amino acid residues 494-651. GCN5 also contains a bromo domain, between amino acids 757-837, which binds to acetylated lysine residues (Smith et al. 1998). In the present study, we showed that both N-terminal region (GCN5N) and C-terminal region (GCN5C) of GCN5 interact with Smad3 in a TGF-β-dependent fashion, suggesting that GCN5 binds to Smad3 through at least two regions in GCN5. This is in contrast to PCAF, which binds to Smad3 through its C-terminal region (Itoh et al. 2000). However, this finding is not surprising because p300 also interacts with Smad3 through multiple regions (Nishihara et al. 1998). GCN5 and PCAF are structurally similar, especially in their C-terminal regions, which contain the HAT and bromo domains. The similarity of the C-terminal region is 83.1%, whereas that of the N-terminal region is only 63.3%. This data adds support to our suggestion that the N-terminal region of GCN5, but not of PCAF, affects the interaction with R-Smads. It will be interesting to
determine the role of N-terminal region of GCN5 and PCAF in Smad signaling pathway in the future.

Multiple transcriptional co-activators appear to be incorporated into Smad transcriptional complexes. Among these co-activators, GCN5 and PCAF are similar in their structures, acetyltransferase activity and substrate specificity. Both of the co-activators are expressed ubiquitously in adult mammalian tissues, but their expression profiles are distinct, although likely to be complementary, in many tissues (Xu et al. 1998). In contrast to PCAF, however, GCN5 is able to facilitate both TGF-β and BMP signaling, suggesting the differences in physiological functions between GCN5 and PCAF in vivo. p300 has also been shown to interact with Smads and facilitate transcription induced by TGF-β (Feng et al. 1998; Janknecht et al. 1998; Nishihara et al. 1998; Shen et al. 1998). However, it is currently not known whether loss of p300/CPB results in perturbations of TGF-β signaling. In the present study, however, we have shown that loss of expression of GCN5 resulted in significant decrease in the transcription induced by TGF-β. Thus, our findings strongly suggest that GCN5 is an essential component in transcriptional regulation induced by TGF-β in certain cells. In the future, it will be interesting to determine how the balance of the multiple transcriptional co-activators regulates the response of cells to TGF-β superfamily proteins.

Experimental procedures
Cell culture—COS7 cells, R mutant Mv1Lu cells (R4-2 cells), 293T cells, and HaCaT human keratinocyte cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics. C2C12 cells were cultured in DMEM containing 20% FBS and antibiotics. To establish stable transfectants, HaCaT cells were transfected with pcDNA3-FLAG-GCN5 by Effectene transfection reagent (Qiagen) and cultured in the presence of 1 mg/ml of G418 sulfate (Gibco) for transfectant selection. After selection, FLAG-GCN5 transfectants were maintained in DMEM containing 10% FBS and 0.5 mg/ml of G418 sulfate. Nuclear extract from MCF-7 cell was prepared as previously described (Kitagawa et al. 2003).

Plasmid constructions—The original constructions of constitutively active forms of TGF-β type I and BMP type IB receptors (TBR-I(TD) and BMP-IB(QD), respectively), and the Smad1, Smad2, Smad3, Smad4, Smad5, Smad8, and Smad3 deletion mutants, were previously described (Nishihara et al. 1998; Kawabata et al. 1998). The expression vector for full-length GCN5 was previously described (Yanagisawa et al. 2002). Deletion mutants of GCN5 were generated by a polymerase chain reaction (PCR)-based approach from a full-length GCN5 cDNA. The expression vector for PCAF was kindly provided by Dr. Itoh (Itoh et al. 2000).

DNAP—MCF-7 cell nuclear extract was pre-cleared with 30 pmol of biotinylated mutated 3xCAGA and 50 μg of Dynabeads M-280 streptavidin (Dynal) at 4°C for 30 min. The supernatant was collected and incubated with 30 pmol of biotinylated wild-type or mutated 3xCAGA and 12 μg of poly(dI-dC) at 4°C overnight. DNA-
bound proteins were precipitated with 100 μl of Dynabeads for 30 min at 4°C, washed, separated with SDS-PAGE, and the resulting SDS-PAGE gel silver-stained. The sequences of wild type 3xCAGA are: 5’-TCGAGAGCCAGACAAGGAGCCAGACAAGGAGCCAGACACTCGAG-3’ (sense strand) and 5’-CTCGAGTGTCTGGCTCTTGCTTGGCTCTCGA-3’ (antisense strand) (Nishihara et al. 1999). The sequences of mutated 3xCAGA are: 5’-TCGAGAGCTACATAAAAAGCTACATATTTAGCTACATACTCGA-3’ (sense strand) and 5’-AGCTCTCGATGTATTTTTCGATGTATAAATCGATGTATGAGCT-3’ (antisense strand).

**Protein identification**—Selected protein-containing bands were excised from silver-stained SDS-PAGE gels, digested in-gel with trypsin, and subjected Autoflex (Bruker), as essentially described (Kanamoto et al. 2002). The searches for protein identification using the obtained peptide mass spectra were performed in the NCBInr sequence database using ProFound (http://prowl.rockefeller.edu/cgi-bin/profound).

**Transfection, immunoprecipitation, and immunoblotting**—COS7 cells were transiently transfected using FuGENE6 transfection reagent (Roche Applied Science), and were then lysed with Nonidet P-40 lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40). Immunoprecipitation and immunoblotting were performed as previously described (Ebisawa et al. 2001).

**ChIP and real-time PCR**—FLAG-GCN5 transfected cells (1x10^6 cells) were cross-linked by addition of 1% formaldehyde for 10 min and glycine added (0.125 M final) for 5 min to stop the cross-linking reaction. Soluble chromat in was prepared using ChIP assay kit (Upstate) according to the manufacturer’s recommendations, and immunoprecipitated with either anti-FLAG M2 antibody (Sigma) or anti-Smad2/3 antiserum (Nakao et al. 1997). Following washes and elution, precipitates were heated overnight at 65°C to reverse cross-linking. DNA fragments were purified using the QIAquick PCR purification kit (Qiagen). Quantitative real-time PCR analysis was performed with an ABI PRISM 7000 sequence detection system (Applied Biosystems), using a SYBR Green PCR master mix (Applied Biosystems). Optimal PCR conditions were found to be 50°C for 2 min and 95°C for 10 min, followed by 45 cycles of PCR consisting of 15 sec at 95°C, and 1 min at 60°C. Specific primer pairs were designed to amplify a target sequence within the human PAI-1 promoter (5’-GCAGGACATCCGGGAGAGA-3’ and 5’-CCAATAGCCTGCCGTGAGA-3’), and the human GAPDH gene, an external standard (5’-GCACCCACACTGCTTTAGCA-3’ and 5’-CAGATAACAAAGTTTGATCGAT-3’).

**Luciferase assay**—R4-2 cells or 293T cells were transiently transfected with an appropriate combination of GaI4-M1-Lux (Itoh et al. 2000), (CAGA)_9-MLP-Lux (Dennler et al. 1998), or Id-1-MLP-Lux promoter-reporter constructs
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(Korchynskyi & ten Dijke 2002), expression plasmids, and pcDNA3. Total amounts of transfected DNAs were the same in each experiment. Luciferase activity was measured by a dual-luciferase reporter assay system (Promega), and values were normalized by Renilla luciferase activity.

RNAi—RNAi was performed as previously described (Kisielow et al. 2002). Briefly, RNAi oligonucleotides were introduced into 293T cells using the Lipofectamine2000 reagent (Invitrogen), with 100 pmol of oligonucleotides and 5 μl of transfection reagent/well in a 12-well tissue culture plate, according to the manufacturer’s introductions. RNA oligonucleotides corresponding to GCN5 (forward: 5'-AAGGAAGAGGACACAGACACC-3'; reverse: 5'-AAGGAAGAGGACACAGACACC-3') were synthesized (Dharmacon). BLAST analysis was used to confirm that negative control RNAi oligonucleotides were not complementary to any mammalian mRNA sequence. 293T cells were transfected with RNAi oligonucleotides and (CAGA)$_9$-MLP-Lux promoter-reporter or FLAG-GCN5 constructs using Lipofectamine2000 reagent.

Reverse transcription and real-time PCR—Total cellular RNA was extracted using Trizol reagent (Invitrogen), and cDNA was synthesized using Superscript III first-strand synthesis system (Invitrogen). Quantitative real-time PCR analysis was performed as described above. The primer sequences used were as follows; human GCN5, forward: 5'-CTGAAGACCATGACTGAGCGG-3', human GCN5, reverse: 5'-TCGGCCACAAAGAGCTTCC-3'; and human GAPDH, forward: 5'-GAAGGTGAAGGTCGGAGTC-3', human GAPDH, reverse: 5'-GAAGATGGTGATGGGATTTC-3'.

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specific response elements in the Id1 promoter. J. Biol. Chem. 277, 4883-4891.


Figure legends

Figure 1. Purification and identification of proteins interacting with activated Smad complexes. A, identification of CAGA-binding proteins in MCF-7 nuclear extract. The nuclear extract was purified with DNAE, separated by SDS-PAGE and then silver-stained. Proteins that bound to wild-type CAGA (WT, lane 1), but not to mutated CAGA (Mut, lane 2), were examined by mass spectrometry analysis. The protein indicated by the arrow was identified as GCN5. B, association of GCN5 with CAGA via activated Smad3. 6Myc-GCN5, Myc-Smad3, and constitutively active TGFR-β receptor (TβR-I (TD)) were transfected into COS7 cells as indicated, and total cell lysates were incubated with biotinylated wild type (WT) or mutated (Mut) 3xCAGA and collected using Dynabeads. DNA affinity purified precipitants (top panel) or aliquots of total cell lysates (lower two panels) were subjected to immunoblotting using anti-Myc, anti-FLAG or anti-HA antibody, respectively.

Figure 2. Binding of GCN5 to the human PAI-1 promoter. A, a schematic diagram of the human PAI-1 promoter. A specific pair of primers covering nucleotides -280 to -25 was designed to amplify the CAGA region located at -280 to -272. B, expression of FLAG-GCN5 in HaCaT stable transfectants. Exogenous expression of FLAG-GCN5 was detected in a HaCaT cell line constitutively expressing FLAG-GCN5 (HG15, lane 1) compared with wild-type HaCaT (WT, lane 2) by FLAG immunoprecipitation followed by immunoblotting using anti-GCN5 antibody. C, TGFB-dependent recruitment of Smad2/3 and FLAG-GCN5 to the PAI-1 promoter. Soluble chromatin was prepared from HG15 cells untreated or treated with TGF-β3 (40 pM) for 1 h, and immunoprecipitated with Smad2/3 antiserum (left panel) or anti-FLAG M2 antibody (right panel). Final DNA extracts were amplified using the primer pair described above (panel A). The data shown are the means of quantitative real-time PCR experiments, performed in triplicate and normalized to human GAPDH gene expression levels.

Figure 3. Physical interaction of GCN5 or PCAF with Smads. A and B, physical interaction of Smads with GCN5 (A) or PCAF (B) was investigated in mammalian cells. COS7 cells were transfected with indicated expression plasmids, and interaction of Smads with GCN5 or PCAF was examined by FLAG-immunoprecipitation of Smads followed by Myc-immunoblotting of GCN5 (A, top panel) or PCAF (B, top panel). Expression levels of each protein were determined by immunoblotting using FLAG, Myc, or HA antibodies (A and B, lower three panels). C, GCN5-interaction domain in Smad3. A schematic representation of Smad3 deletion mutants is shown (bottom scheme). The GCN5-interaction domain of Smad3 was determined by immunoprecipitation and immunoblotting, as described in panel A (top). The lower three panels show expression levels of transfected proteins. D, Smad3-interaction regions in GCN5. Structures of GCN5 deletion mutants used are shown (bottom scheme). Smad3-interaction regions were examined by immunoprecipitation and immunoblotting as described in panel A (top). Expression levels of transfected proteins are shown in the lower three panels.
Figure 4. Enhancement of TGF-β and BMP signaling by GCN5. A, potentiation of transcriptional activity of Gal4-Smad3 by GCN5. R mutant Mv1Lu (R4-2) cells were co-transfected with Gal4-M1-lux and the indicated expression plasmids, and luciferase activities were determined. B and C, transcriptional activity induced by TβR-I(TD) (B) or TGF-β3 (40 pM) (C) using (CAGA)$_9$-MLP-lux. C2C12 cells (B) or 293T cells (C) were transfected with the indicated plasmids and luciferase activities were determined. +, ++, and +++ are 0.05, 0.1, and 0.2 µg of DNA, respectively, transfected into cells. D, dominant-negative effect of amino-terminal region of GCN5. The effects of GCN5 amino-terminal deletion mutant (N) on (CAGA)$_9$-MLP-lux transcription induced by TβR-I were examined. The GCN5 deletion mutant shown in Figure 3D was used. +, ++, and +++ are 0.05, 0.1, and 0.2 µg of DNA, respectively, transfected into R4-2 cells. E, transcriptional effects of GCN5 and PCAF on Id1-MLP-lux transcription induced by BMPR-IB(QD). C2C12 cells were transfected with the plasmids indicated, and luciferase activities were determined. +, ++, and +++ are 0.05, 0.1, and 0.2 µg of DNA, respectively, transfected into C2C12 cells.

Figure 5. Effect of decreased expression level of GCN5 on TGF-β signaling. A and B, effect of RNAi specific for GCN5 on endogenous expression level of mRNA for GCN5 (A) or protein for GCN5 (B). 293T cells were transfected with RNAi oligonucleotides corresponding to GCN5 without (A) or with (B) an expression plasmid for FLAG-GCN5. Twenty-four hours after transfection, GCN5 mRNA or protein was analyzed by real-time PCR (A) or by immunoblotting using anti-FLAG M2 antibody (B), respectively. C, repression of TGF-β-dependent transactivation in the presence of RNAi for GCN5. 293T cells were transfected with RNA oligonucleotides of the indicated RNAi, and (CAGA)$_9$-MLP-lux. Cells were treated with 40 pM TGF-β3 for 24 h and luciferase activity was determined.
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A Human PAI-1 promoter

\[
\begin{align*}
&\text{CAGA} \quad \text{CAGA} \quad \text{CAGA} \quad \text{TATA} \\
&-730 \quad -581 \quad -280 \quad -28
\end{align*}
\]

Amplified region

B

FLAG-GCN5

HG15  WT

IP; FLAG
Blot. anti-GCN5

C

IP; anti-Smad2/3

IP; anti-FLAG

Relative Smad2/3 binding

Relative FLAG-GCN5 binding

TGF-β

TGF-β
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### Table A

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**Legend:**
- **IP:** IP; FLAG Blot; Myc
- **6Myc-GCN5**

### Table B

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**Legend:**
- **IP:** IP; FLAG Blot; Myc
- **6Myc-PCAF**

**Diagram:**
- **A:** 6Myc-GCN5
- **B:** 6Myc-PCAF

**Notes:**
- Smad 1, Smad 6, and Smad 7 not shown in the table.
- The diagram shows the results of immunoprecipitation (IP) experiments with Myc and FLAG tagging.
Kahata et al. Fig.3

**C**

<table>
<thead>
<tr>
<th>6Myc-GCN5</th>
<th>FLAG-Smad3</th>
<th>TβR-I(TD)-HA</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>+</td>
<td>A</td>
<td>B</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>D</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

- IP; FLAG Blot; Myc
- IP; (-) Blot; Myc
- IP; FLAG Blot; FLAG
- IP; (-) Blot; HA

**D**

<table>
<thead>
<tr>
<th>6Myc-GCN5</th>
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<th>N</th>
<th>C</th>
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<td>-</td>
<td>-</td>
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<tr>
<td>+</td>
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- IP; FLAG Blot; Myc
- IP; (-) Blot; Myc
- IP; FLAG Blot; FLAG
- IP; (-) Blot; HA

<table>
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<tr>
<th>Full</th>
<th>361</th>
<th>494</th>
<th>651</th>
<th>757</th>
<th>837</th>
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</thead>
<tbody>
<tr>
<td>N</td>
<td>HAT domain</td>
<td>Bromo domain</td>
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<td></td>
</tr>
</tbody>
</table>
Kahata et al. Fig. 5

A

B

C

(CAGA)₉-MLP-Lux

[Graph showing GCN5/GAPDH ratios for siRNA and negative control vs. GCN5]

<table>
<thead>
<tr>
<th>siRNA</th>
<th>Negative control</th>
<th>GCN5</th>
</tr>
</thead>
<tbody>
<tr>
<td>FLAG-GCN5</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

Blot: FLAG

FLAG-GCN5

Luciferase activity (arbitrary unit)

[Graph showing Luciferase activity for TGF-β (-) and TGF-β (+) with siRNA control and GCN5]