Ubc9 interacts with chicken ovalbumin upstream promoter-transcription factor I and represses receptor-dependent transcription

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Abstract

Chicken ovalbumin upstream promoter-transcription factors (COUP-TFs) are orphan receptors involved in regulation of neurogenesis and organogenesis. COUP-TF family members are generally considered to be transcriptional repressors and several mechanisms have been proposed to underlie this activity. To explore novel transcriptional coregulators for COUP-TFs, we used the COUP-TFI as bait in a yeast two-hybrid screen of an adrenocortical adenoma cDNA library. We have identified Ubc9, a class E2 conjugating enzyme of small ubiquitin-related modifier (SUMO)-1 as a COUP-TFI corepressor. Ubc9 interacts with COUP-TFI in yeast and in glutathione S-transferase pulldown and coimmunoprecipitation assays. Fluorescence imaging studies show that both Ubc9 and COUP-TFI are colocalized in the nuclei of transfected COS-1 cells. The C-terminal region of Ubc9 encoding amino acids 59–158 interacts with the C-terminus of COUP-TFI encoding amino acids 383–403, in which transcriptional repression domains are located. Mammalian one-hybrid assays utilizing a variety of Ubc9 fragments fused to Gal4 DNA-binding domain show that a Ubc9 fragment encoding amino acids 1–89 contains autonomous transferrable repression domain. Transfection of Ubc9 into COS-1 cells markedly enhances transcriptional repression by Gal4 DNA-binding domain-fused to COUP-TFI(155–423), but not by Gal4–COUP-TFI(155–388) which lacks a repressor domain. Coexpression of a C-terminal deletion mutant of Ubc9(1–58), which fails to interact with COUP-TFI, but retains a transcriptional repression domain, has no effect on Gal4–COUP-TFI-mediated repression activity. These findings indicate that interaction of Ubc9 with COUP-TFI is crucial for the corepressor function of Ubc9. Overexpression of Ubc9 similarly enhances COUP-TFI-dependent repression of the promoter activity of the bovine *CYP17* gene encoding steroid 17 α -hydroxylase. In addition, the C93S mutant of Ubc9, which abrogates SUMO-1 conjugation activity, continues to function as a COUP-TFI corepressor. Our studies indicate that Ubc9 functions as a novel COUP-TFI corepressor, the function of which is distinct from its SUMO-1 conjugating enzyme activity.

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Introduction

Chicken ovalbumin upstream promoter-transcription factors (COUP-TFs) are orphan members of the nuclear receptor superfamily for which a ligand has yet to be identified (Wang *et al.*

1989, Ladias & Karathanasis 1991, Malik & Karathanasis 1995, Tsai & Tsai 1997). COUP-TFI/EAR3 (encoded by *NR2F1*) and COUP-TFII/ARP-1 (encoded by *NR2F2*) are closely related transcription factors in mammals and are involved in the regulation of crucial biological

processes, such as neurogenesis, organogenesis and determination of cell fate (Pereira *et al.* 1995, Tsai & Tsai 1997, Cooney *et al.* 2001).

Although COUP-TF was originally identified as an activator of the chicken ovalbumin gene, COUP-TF family members are generally considered to be repressors of ligand-activated nuclear receptors, such as retinoic acid (RAR), thyroid hormone (TR), oestrogen and vitamin D₃ receptors (VDR), and peroxisome proliferator-activated receptor α (Cooney *et al.* 1992, Tsai & Tsai 1997). Several mechanisms have been proposed to explain the COUP-TF-mediated repression activity (Berrodin *et al.* 1992, Klierer *et al.* 1992*a,b*, Tran *et al.* 1992, Cooney *et al.* 1993, Casanova *et al.* 1994, Leng *et al.* 1996, Achatz *et al.* 1997, Shibata *et al.* 1997*a*, 2003*b*). COUP-TFs are able to bind to a variety of direct repeats, such as DR1, DR3, DR4 and DR5 of the AGGTCA motif, which are recognized by RAR, THR and VDR. This ability makes COUP-TFs capable of competing for the response elements of these receptors, thus acting as passive repressors of the transcriptional activation induced by them. Another mechanism of passive repression by COUP-TFs involves their ability to heterodimerize with the 9-*cis* retinoic acid receptor, reducing its availability for other nuclear receptors that use it as a partner. COUP-TFs also function as repressors by quenching transactivator-dependent transcription and transrepression of activated transcription. In addition, COUP-TFs, like THR and RAR, are able actively to repress the basal promoter activity of several target genes, through interaction with corepressors such as nuclear receptor corepressor (N-CoR) and silencing mediator for retinoid and thyroid hormone receptors (SMRT) in mammalian cells (Bailey *et al.* 1997, Shibata *et al.* 1997*a*). Therefore, COUP-TFI can function as a repressor *in vivo* by utilizing corepressors that are common for members of the THR and RAR subfamily.

Our recent studies have suggested that COUP-TFs and steroidogenic factor-1 (SF-1) have key roles in the transcriptional regulation of steroidogenic *P450* genes (Shibata *et al.* 1998, 2000*a,b*, 2001, Shibata *et al.* 2003*a,b*). Transfection studies showed that SF-1 activated and COUP-TFs repressed bovine *CYP17* gene transcription as a result of mutually exclusive binding to the promoter in mouse adrenocortical Y-1 cells. In addition, levels of expression of COUP-TFI have

been shown to be highly correlated with those of N-CoR, but inversely correlated with those of CYP17 in human adrenocortical adenomas, indicating that COUP-TFI may play a crucial part in the regulation of steroidogenesis in adrenocortical adenomas. To search for novel coregulator proteins for COUP-TFI, we screened COUP-TF-interacting proteins from a human adrenocortical tumour cDNA library with a yeast two-hybrid system and a COUP-TFI fragment encoding amino acids 55–423 as bait. We then identified Ubc9 (Kovalenko *et al.* 1996, Wang *et al.* 1996), the human homologue of yeast class E2 small ubiquitin-related modifier (SUMO)-1-conjugating enzyme.

SUMO-1 (Melchior 2000, Hay 2001, Jackson 2001, Muller *et al.* 2001, Seeler & Dejean 2001, Wilson & Rangasamy 2001) is known to be covalently conjugated to a variety of cellular substrates, and this modification utilizes a three-step enzymatic pathway analogous to the pathway of ubiquitin conjugation. The enzymes for these three steps are Aos1-Uba2 as the activating enzyme (class E1), Ubc9 as the conjugating enzyme (class E2), and PIAS 1 (Hochstrasser 2001, Jackson 2001, Kahyo *et al.* 2001), RanBP2 (Pichler *et al.* 2002) and PC2 (Kagey *et al.* 2003) as the SUMO-1 ligase (class E3), which has recently been identified. Ubc9 also has several biological functions and not only catalyses SUMO-1 conjugation of different proteins, but also is particularly crucial in the regulation of transcription. Available data currently implicate SUMO in the regulation of protein–protein interactions (Matunis *et al.* 1998), subcellular nuclear localization (Seeler & Dejean 2001, Pichler & Melchior 2002), protein–DNA interactions (Goodson *et al.* 2001) and enzymatic activity (Hardeband *et al.* 2002), and there is also evidence that SUMO can act as an antagonist of ubiquitin (Desterro *et al.* 1998, Hoege *et al.* 2002). Recent reports indicate that SUMO modification provides not merely modulatory but actually inhibitory domain function (Iniguez-Lluhi & Pearce 2000, Kim *et al.* 2002, Yang *et al.* 2002); indeed, it is notable that several well-characterized repressors contain SUMO consensus sites within their repression domains.

In the present study, overexpression of Ubc9 in transfected COS-1 cells enhanced COUP-TFI-mediated repression activity of the bovine *CYP17* promoter in a trichostatin A-insensitive manner.

Figure 1 Diagram of various deletion mutants of (A) COUP-TFI and (B) Ubc9. (A) The COUP-TFI fragments shown were cloned into the pGBKT7, pcDNA3.1/His and pCMV-BD expression vectors as described in Materials and methods. Based on other nuclear hormone receptors as a reference, the sequences from amino acids 1–85, 86–149, 150–183 and 184–423 have been designated domains A/B, C, D and E/F, respectively. (B) The Ubc9 fragments shown were cloned into the pGADT7, pcDNA3.1/His and pCMV-BD expression vectors as described in Materials and Methods.

The corepressor activities of Ubc9 were not abolished by the C93S substitution that abrogates SUMO-1 conjugating enzyme activity. We propose that Ubc9 can function as a novel COUP-TFI corepressor, the function of which is independent of SUMO-1 conjugating enzyme activity.

Materials and methods

Plasmid constructs

The expression plasmids pRSV-COUP-TFI, pFL-COUP-TFI, pFL-COUP-TFII, pRSV-Gal4-COUP-TFI (155–423), pRSV-Gal4-COUP-TFIΔ35, pRSV-Gal4(1–147), pRSV-Gal4-VP16, pAS1 cyh2-TRβ168–456 and p4 × Gal4-tk-Luc have been described previously (Leng *et al.* 1996, Shibata *et al.* 1997a). pGBT9-Ad4BP/SF-1 and pGBT9-DAX-1 were generous gifts from Dr K Morohashi (National Institute for Basic Biology, Japan).

A diagram of various deletion mutants of COUP-TFI and Ubc9 is shown in Fig. 1.

The Gal4 DBD-COUP-TFI(55–423) yeast expression plasmid [pGBKT7-COUP-TFI(55–423)] was constructed by inserting the SmaI-EcoRI blunt-ended fragment of pFL-COUP-TFI into the SmaI site of pGBKT7 vector (Clontech, Palo Alto, CA, USA). pGBKT7-COUP-TFI(55–315) was constructed by SalI digestion of pGBKT7-COUP-TFI(55–423) followed by self-ligation with T4 DNA ligase. The COUP-TFI deletion mutant series COUP-TFI(378–423), COUP-TFI(315–423), COUP-TFI(154–403), COUP-TFI(154–383), COUP-TFI(154–363) and COUP-TFI(1–403) were generated by PCR, introducing flanking restriction enzyme sites, thereby subcloning into the pGBKT7 yeast expression vector (Clontech). pGBKT7-COUP-TFII was constructed by inserting the EcoRI fragment of pFL-COUP-TFII into the EcoRI site of pGBKT7 vector. pGADT7-Ubc9(1–158) was cloned as a COUP-TFI-interacting protein by using a yeast two-hybrid system. pGADT7-Ubc9(1–58) was constructed by PmeI-SpeI digestion (filled) of pGADT7-Ubc9(1–158), followed by ligation with T4 DNA ligase.

pGADT7-Ubc9(C93S) was constructed with a site-directed mutagenesis kit. Mutagenesis of Ubc9 was performed with the Transformer Site Directed Mutagenesis kit (Clontech) and the mutagenesis oligonucleotide 5'-CTTCGGGGACAGTGTCCCTGTCCATCTTAGAGG-3'. The underlined sequence corresponds to the Cys (TGC) to Ser (TCC) codon change. The Ubc9 deletion mutant series Ubc9(1-75), Ubc9(1-89), Ubc9(1-100), Ubc9(59-158), Ubc9(76-158), and Ubc9(90-158) were generated by PCR, introducing flanking restriction enzyme sites, thereby subcloning into the pGADT7 yeast expression vector (Clontech) and pCMV-BD mammalian expression vector (Stratagene, La Jolla, CA, USA). pcDNA3.1/His-Ubc9(1-158), pcDNA3.1/His-Ubc9(C93S) and pcDNA3.1/His-Ubc9(1-58) were constructed by inserting the EcoRI-XhoI fragment of pGADT7-Ubc9(1-158), pGADT7-Ubc9(C93S) and pGADT7-Ubc9(1-58), respectively, into the EcoRI-XhoI site of pcDNA3.1/His vector (Invitrogen). pCMV-BD-Ubc9(1-158), pCMV-BD-Ubc9(C93S), and pCMV-BD-Ubc9(1-58) were constructed by inserting the EcoRI-PstI fragments of pGADT7-Ubc9(1-158), pGADT7-Ubc9(C93S) and pGADT7-Ubc9(1-58) into the EcoRI-PstI site of pCMV-BD vector (Stratagene). pGL3-3 CRS2 was constructed by ligating cDNA which was made by annealing two oligonucleotides, 5'-CCCCAAGTCAAGGAGAAGGTCAGGGAAAGTCAAGGAGAAGGTCAGGGAAAGTCAAGGAGAAGGTAGGGCCGC-3' and 5'-TCGAGCGGCCCTGACCTTCTCCTTGACTTTCCCTGACCTTCTCCTTGACTTTCCCTGACCTTCTCCTTGACTTTGGGGTAC-3', into the KpnI-XhoI site of pGL3 promoter vector (Promega). pEGFP-Ubc9 and pEGFP-Ubc9(C93S) were constructed by inserting the EcoRI-XhoI fragments of pGADT7-Ubc9 and pGADT7-Ubc9(C93S), respectively, into the EcoRI-SalI site of pEGFP-C2 vector (Clontech). pDsRed-COUP-TFI was generated by PCR, introducing the flanking EcoRI-BamHI site, thereby subcloning into the pDsRed vector (Clontech). The Ubc9(1-158), Ubc9(C93S) and Ubc9(1-58) were subcloned into the EcoRI-XhoI site of pGEX4T-1 vector (Amersham Pharmacia Biotech).

DNA sequencing of all the constructs was confirmed by ABI PRISM dye terminator cycle sequencing analysis (Amersham Pharmacia Biotech).

Cloning of Ubc9 by a yeast two-hybrid system

Yeast two-hybrid screening was conducted with the MATCHMAKER Two-Hybrid System 3 kit (Clontech) and COUP-TFI (amino acids 55-423) as bait. A human adrenocortical tumour cDNA library was prepared as follows. Messenger RNA was prepared from a cortisol-producing adrenocortical adenoma with an Oligotex-dT30mRNA purification kit (TaKaRa Bio Inc, Tokyo, Japan), and cDNA was synthesized by reverse transcription of the mRNA with an oligo (dT)18 linker. We then transformed *E. coli* competent cells with the cDNAs by electroporation, and ligated the cDNAs into the pGADT7 vector. Yeast strain AH109 containing pGBKT7-COUP-TFI(55-423) was transformed with a human cortisol-producing adrenocortical adenoma cDNA library in pGADT7 (Clontech) and plated on synthetic complete medium lacking tryptophan, adenine, leucine and histidine, as described by Durfee *et al.* (1993).

His⁺ and Ade⁺ colonies exhibiting β -galactosidase activity by filter lift assay were further characterized according to the manufacturer's procedure (Clontech). β -galactosidase activity was determined with chlorophenol red β -D-galactopyranoside as described previously (Shibata *et al.* 1997a). To recover the library plasmids, total DNA from the yeast was isolated with a Zymoprep Yeast Plasmid Miniprep kit (Zymo Research, Orange, CA, USA) and used to transform *E. coli* (HB101) in the presence of ampicillin. To ensure that the correct cDNAs were identified, the library plasmids isolated were transformed into Y187 containing pGBKT7-COUP-TFI(55-423) and β -galactosidase activity was determined. The specificity of the interaction of #2-4 (Ubc9 1-158), one of the 20 positive clones, with COUP-TFI was determined by mating with Y187, which contains pGBKT7-lamin (Clontech). The β -galactosidase activity of these diploids was examined by the filter lift and chlorophenol red β -D-galactopyranoside methods. The sequence of the #2-4 clone was identical to that of Ubc9, the human homologue of yeast ubiquitin-conjugating enzyme, that had been submitted to GenBank. The yeast two-hybrid system was also used to determine interaction of Ubc9 with COUP-TFI and other nuclear receptors (Fig. 2).

Mammalian cell culture, transient transfections and luciferase assays

COS-1 cells were routinely maintained in DMEM (Life Technologies) supplemented with 10% fetal bovine serum (Life Technologies). Twenty-four hours before transfection, 5×10^4 cells per well of a 24-well dish were plated in the medium. All transfections were carried out using TransFast (Promega) with 0.3 µg/well of the luciferase reporter, 0.03 µg/well of pRL-TK internal control plasmids and the indicated amounts of expression plasmids as described previously. Cells were transfected with the DNAs indicated by using TransFast (Promega) for each well of the 24-well dish according to the manufacturer's guidelines. Cells were harvested 48 h after transfection, and cell extracts were assayed for both Firefly and Renilla luciferase activities with a Dual-Luciferase Reporter Assay System (Promega). Relative luciferase activity was determined as ratio of Firefly/Renilla luciferase activities, and data are expressed as the mean ($\pm$ S.D.) of triplicate values obtained from a representative experiment that was independently repeated at least three times.

Glutathione S-transferase pulldown assay

Glutathione S-transferase (GST) (pGEX4T-1) protein and GST-Ubc9 [pGEX4T-1-Ubc9(1–158)] fusion protein were expressed and extracted in *E. coli* HB101 as described previously (Shibata *et al.* 1997a). GST pulldown assay was performed as described, with modifications: 50 µl glutathione-sepharose beads 4B (Amersham Pharmacia Biotech) stored in beads incubation buffer (50 mM potassium phosphate buffer pH 7.4, 100 mM NaCl, 1 mM MgCl₂, 10% glycerol and 0.1% Tween 20) were incubated with bacterial extracts containing GST fusion proteins together with beads incubation buffer for 30–60 min at room temperature. Preparation of bacterial extracts containing GST fusion protein was as described previously (Shibata *et al.* 1997a). The supernatant was then removed, and the beads were washed twice with beads incubation buffer. *In vitro*-translated and -radiolabelled proteins were obtained by using TNT Coupled Reticulocyte Lysate Systems (Promega). Crude lysates were incubated with the beads in 200 µl beads incubation buffer for 30 min at room temperature with a circle rotator.

Finally, the beads were washed five times with 1 ml beads incubation buffer and the proteins were solubilized in SDS loading buffer and analysed on SDS-PAGE (12.5% polyacrylamide gel). The input lanes contained 20% of the labelled protein used for binding.

Coimmunoprecipitation assay

The cells were lysed with lysis buffer [10 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% Triton X-100, 5 mM EDTA, 2 mM phenyl methylsulphonyl fluoride (PMSF)], and western blots were performed before the immunoprecipitation steps. The same samples for the western blots were diluted to 1 ml in immunoprecipitation buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 10 mM dithiothreitol, 5 ng/µl aprotinin, 0.5 mM PMSF, 0.1% Tween 20) and precleared with protein G plus-agarose beads (Santa Cruz Biotechnology, Santa Cruz, CA, USA); antibodies were then added for 1 h. Immune complexes were adsorbed to protein G plus-agarose beads and washed four times in immunoprecipitation buffer. Proteins were then separated on 12.5% polyacrylamide gels and transferred onto Hybond enhanced chemiluminescence (ECL) nitrocellulose membranes (Amersham Pharmacia Biotech). The primary antibodies used for immunoprecipitation were rabbit polyclonal anti-COUP-TFI antibody (a generous gift from Dr Ming-Jer Tsai, Baylor College of Medicine, Houston, TX, USA) (Wang *et al.* 1989); those used for the western blots were anti-COUP-TFI or anti-Xpress mouse monoclonal antibodies (Invitrogen).

Fluorescence imaging

COS-1 cells were transiently transfected with expression vectors of pEGFP-Ubc9, and pDsRed-COUP-TFI. Live cell microscopy of GFP fusion and DsRed fusion proteins was performed on a confocal microscope (Axiovert 100 M, Carl Zeiss Co., Ltd). Imaging for GFP and DsRed was performed by excitation at 488 nm and 543 nm respectively from an argon laser, and the emissions were viewed through band passes ranging from 500 to 550 nm and 550 to 600 nm respectively, by band-pass regulation with LSM510 (Carl Zeiss Co., Ltd). All images were processed as tiff (tagged

image file format) on Photoshop 6.0 using standard image processing techniques.

Northern blot analysis

The human tissue northern blots were obtained from Clontech and hybridized at 42 °C overnight with ³²P-labeled cDNA probes of the full-length 1.1 kb hUbc9, full-length 1.3 kb hCOUP-TFI, or 1.1 kb GAPDH (Clontech) cDNAs according to the manufacturer's recommendation. The membranes were washed at a final stringency of 0.1 × SSC–0.1% SDS at 50 °C and analysed with a BAS 3000 image scanner (Fuji Film Co.). The mRNA levels were determined by comparison with GAPDH mRNA levels.

Western blot analysis

Whole-cell lysates were prepared by homogenization in buffer composed of 10 mM Tris–HCl pH 8.0, 150 mM NaCl, 1% Triton X-100, 5 mM EDTA and 2 mM PMSF. After separation by electrophoresis on 12.5% polyacrylamide gels, the proteins were electroblotted onto nitrocellulose membranes (Hybond ECL, Amersham Pharmacia Biotech) at 200 V. The membranes were then incubated with the following primary antibodies: rabbit polyclonal anti-COUP-TFI antibody (Wang *et al.* 1989) and mouse monoclonal anti-Xpress IgG (Invitrogen). They were then incubated with horseradish-peroxidase-labeled donkey anti-rabbit IgG (Amersham Pharmacia Biotech) or donkey anti-mouse IgG (Amersham Pharmacia Biotech) secondary antibodies for each of the above, and visualized with the enhanced chemiluminescence western blotting system (Amersham Pharmacia Biotech).

Statistics

All experiments were performed in triplicate several times. The error bars in the graphs of individual experiments correspond to the S.D. of the triplicate values.

Results

Identification of Ubc9 as a COUP-TFI-interacting protein by yeast two-hybrid system

In order to identify proteins that might repress the activity of the COUP-TFI, we used COUP-TFI

encoding amino acids 55–423 as bait in a yeast two-hybrid screen with a cDNA library prepared from a human adrenocortical adenoma. In this manner, we identified a full-length clone of Ubc9 that had a 1.1 kb insert containing an entire open-reading frame encoding 158 amino acids. Ubc9 is an enzyme involved in the conjugation of SUMO-1 to target proteins. To demonstrate that Ubc9 interacts specifically with COUP-TFI, we performed a yeast two-hybrid assay. As shown in Fig. 2, Ubc9 interacted with COUP-TFI, and the interaction was specific, as no interaction between Gal4 DBD–COUP-TFI (amino acids 55–423) fusion and Gal4 activation domain (Gal4 AD; empty vector) was observed (Fig. 2A). In contrast, as a positive control, we showed a strong interaction between Gal4 DBD–COUP-TFI and Gal4 AD–COUP-TFI, because COUP-TFI readily forms homodimers (data not shown). In addition, Ubc9 did not interact with unrelated bait corresponding to lamin (data not shown). Besides interacting with COUP-TFI, Ubc9 also interacted with COUP-TFII and SF-1, but not with DAX-1 or unliganded TRβ (Fig. 2A).

To identify interaction domains more precisely, various fragments of COUP-TFI and Ubc9 (Fig. 1) were cotransformed in yeast, and β-galactosidase liquid assays were performed to quantitate the protein–protein interaction (Figs 2B and C). Gal4 DBD–COUP-TFI interacted with most of the N-terminal and C-terminal deletion mutants of Ubc9 except Ubc9(1–58), suggesting that the N-terminal fragment of Ubc9(1–58) was dispensable for interaction with COUP-TFI (Fig. 2B). In addition, introduction of the C93S substitution into the protein-coding sequence of Ubc9, which renders it incapable of conjugation with SUMO-1, had little effect on the interaction of COUP-TFI with Ubc9, indicating that the SUMO-1-conjugating activity of Ubc9 is unnecessary for Ubc9 to interact with COUP-TFI. Subsequently, interaction of several deletion fragments of COUP-TFI with Ubc9 was examined in yeast two-hybrid assays (Fig. 2C). The C-terminal deletion mutant of COUP-TFI encoding amino acids 55–315 did not interact with Ubc9. In addition, both C-terminal COUP-TFI fragments encoding amino acids 315–423 and 378–423 showed significant interaction with Ubc9; however, the interaction between the C-terminal COUP-TFI fragments and Ubc9 (lanes 3 and 4 in Fig. 2C) was

Figure 2 Interaction of Ubc9 with COUP-TFI and several nuclear receptors in yeast two-hybrid assays. (A) Interaction of Ubc9 and various nuclear receptors (COUP-TFI, COUP-TFII, SF-1, DAX-1 and TRβ) in yeast. (B) Interaction of various Ubc9 fragments with COUP-TFI(55–423) in yeast. (C) Interaction of various COUP-TFI fragments with Ubc9 in yeast. β-galactosidase activity was assayed in liquid cultures in three separate experiments, each with triplicate samples. Values are expressed as the average Miller Units (±S.D.) of triplicate values.

not sufficiently strong compared with the interaction between COUP-TFI(55–423) and Ubc9 (lane 1 in Fig. 2C).

COUP-TFI interacts with Ubc9 *in vitro*

To confirm that Ubc9 and COUP-TFI interact directly, we performed *in vitro* GST pulldown assays. The GST protein and its fusion protein GST-Ubc9 were bacterially expressed, purified and incubated with *in vitro*-translated ³⁵S-methionine-labeled COUP-TFI proteins, as described in Materials and methods. As reported previously (Cooney *et al.* 1992), *in vitro*-translated COUP-TFI(1–423) produced two bands, as a result of two different translation start sites (Fig. 3A). Both GST-Ubc9 and GST-Ubc9(C93S) proteins inter-

acted significantly with both COUP-TFI(1–423) and COUP-TFI(55–423), whereas GST-Ubc9(1–58) failed to interact with both COUP-TFI proteins, suggesting that deletion of the N-terminal 54 amino acids has little effect on the interaction of COUP-TFI with Ubc9 and that SUMOylation activity of Ubc9 is not mandatory for interaction with COUP-TFI. Indeed, our attempts to detect a SUMO-1-modified COUP-TFI protein have not yielded results to support the presence of such a COUP-TFI form (data not shown). In contrast, GST alone is unable to interact with wild-type COUP-TFI(1–423) or N-terminal-deleted COUP-TFI(55–423). These results confirmed the interaction results obtained with yeast. In addition, COUP-TFI fragments encoding amino acids 1–403 and 154–403 interacted strongly with GST-Ubc9;

Figure 3 Ubc9 interacts directly with COUP-TFI in GST pull-down assays. (A) Interaction between ³⁵S-labeled COUP-TFI(1–423) or COUP-TFI(55–423) and GST-Ubc9, GST-Ubc9(C93S) or GST-Ubc9(1–58). Input is 20% of the labeled protein used in the assays. The bound ³⁵S-COUP-TFI proteins were visualized by phosphorimaging. (B) Interaction between ³⁵S-labeled COUP-TFI(1–403), COUP-TFI(154–403), COUP-TFI(154–383) or COUP-TFI(154–363) and GST-Ubc9. Input is 20% of the labeled protein used in the assays. The bound ³⁵S-COUP-TFI proteins were visualized by phosphorimaging.

however, COUP-TFI fragments encoding amino acids 154–383 and 154–363 showed little interaction with GST-Ubc9 (Fig. 3B). These findings indicate that the COUP-TFI domain encoding amino acids 383–403 is important for interaction with Ubc9, data that are well compatible with yeast interaction data.

Interaction and subcellular localization of Ubc9 and COUP-TFI in mammalian cells

The association between COUP-TFI and Ubc9 was further investigated by coimmunoprecipitation assays (Figs 4A–C). COS-1 cells were transfected with Xpress-tagged Ubc9 (pcDNA3.1/His–

Ubc9) and RSV promoter-driven COUP-TFI expression vectors (pRSV–COUP-TFI). Polyclonal anti-COUP-TFI antibody was first used to precipitate the protein complexes containing COUP-TFI, and the presence of Ubc9 protein in these complexes was subsequently examined by immunoblotting with a polyclonal anti-Xpress antibody. The presence of Ubc9 protein was detected in lysates from cells transfected with both RSV–COUP-TFI (pRSV–COUP-TFI) and Xpress-tagged Ubc9 (pcDNA31/His–Ubc9), but not with RSV–COUP-TFI or Xpress-tagged Ubc9 alone (Fig. 4A). These findings indicate that COUP-TFI interacts with Ubc9 in mammalian cells. Taken together with interaction data (Figs 2–4), these findings show that Ubc9 interacts specifically and directly with COUP-TFI.

In order to determine if Ubc9 and COUP-TFI could interact within a cellular environment, COS-1 cells were transfected with EGFP, EGFP-tagged Ubc9, EGFP-tagged Ubc9(C93S), DsRed, or DsRed-tagged COUP-TFI alone or in various combinations and photographed using a fluorescence microscope (Figs 4D–I). COS-1 cells transfected with EGFP alone displayed a diffuse green fluorescence (data not shown). EGFP-tagged Ubc9 and EGFP-tagged Ubc9(C93S) showed localization in both nucleus and cytoplasm (Figs 4D and G). COS-1 cells transfected with DsRed alone displayed a diffuse red fluorescence, whereas cells transfected with DsRed-tagged COUP-TFI showed predominantly nuclear localization with occasional dot formation. When cells were then cotransfected with both EGFP-tagged Ubc9 and DsRed-tagged COUP-TFI, expression of both EGFP-Ubc9 and DsRed–COUP-TFI was colocalized in the nucleus and was not altered compared with the expression obtained when either was transfected alone (Figs 4D–F). In addition, expression of both EGFP-tagged Ubc9(C93S) and DsRed–COUP-TFI was colocalized in the nucleus (Figs 4G–I).

COUP-TFI-mediated transcriptional repression is potentiated by Ubc9 independently of the SUMO-1 conjugating activity

To examine the influence of Ubc9 overexpression on COUP-TFI-mediated transcriptional repression, COS-1 cells were transiently transfected with expression plasmids for Gal4 DBD–COUP-TFI and Ubc9 and the reporter construct

Figure 4 Ubc9 and COUP-TFI are associated and colocalized in the nuclei of transfected COS-1 cells. (A–C) Coimmunoprecipitation assays. COS-1 cells were transfected with pRSV-COUP-TFI or pXpress-Ubc9 expression constructs [pcDNA3.1/His-Ubc9(1–158)], or both, and the amount of DNA was kept constant by the addition of empty expression vectors. (A) Whole-cell extracts were subjected to immunoprecipitation (IP) with anti-COUP-TF antibody, and immunoprecipitates were subsequently analysed by immunoblotting (Blot) with anti-Xpress antibody. Immunoblots (Blot) of whole-cell extracts were transfected by various expression constructs as indicated by (B) anti-COUP-TF and (C) anti-Xpress antibodies. (D–I) Subcellular localization of Ubc9 and COUP-TFI. (D–F) EGFP-Ubc9 or (G–I) EGFP-Ubc9(C93S) was cotransfected with DsRed-COUP-TFI in COS-1 cells. These proteins are colocalized in the nuclei of the transfected COS-1 cells.

containing 4 × Gal4 binding sites-thymidine kinase promoter-linked luciferase reporter DNA (4 × Gal4-tk-Luc). Transfection of a Gal4 DBD fused to the COUP-TFI(155–423) cDNA, designated as Gal4-COUP-TFI, significantly repressed basal promoter activity of the reporter gene, as shown previously (lane 5 in Fig. 5A) (Leng *et al.* 1996, Shibata *et al.* 1997a). In addition, coexpression of wild-type Ubc9 or mutant Ubc9(C93S) potentiated the repressor function of Gal4-COUP-TFI in a dose-dependent manner in COS-1 cells (lanes 6–9 in Fig. 5A), whereas coexpression of mutant Ubc9(1–58), which is not able to interact with COUP-TFI, did not affect the reporter activity (lanes 10–11 in Fig. 5A). Ubc9 had no significant effect on the transcription activity of the reporter construct in the absence of Gal4-COUP-TFI (lanes 1–4 in Fig. 5A). The ability of Ubc9 to potentiate the repression activity of COUP-TFI is specific, as coexpression of Ubc9 did not affect the activity of a reporter construct with no Gal4 binding sites (tk-Luc) (data not shown). When Gal4 DNA-binding domain fused to VP16 (Gal4-VP16) was overexpressed, strong activation activity of a

reporter gene (4 × Gal4-tk-Luc) was observed (Fig. 5B). Moreover, overexpression of Ubc9 did not affect the transcription activity of Gal4-VP16 (Fig. 5B), suggesting that Ubc9 does not show non-specific repression activity. These findings suggest that Ubc9 can function as a corepressor for COUP-TFI-mediated repression activity and that SUMO-1-conjugating activity of Ubc9 is not mandatory for COUP-TFI-mediated repression activity. The decrease in reporter gene activity was not the result of an increased cellular concentration of COUP-TFI, because immunoblots did not show alterations in immunoreactive COUP-TFI protein content (data not shown). Coexpression of Ubc9 did not increase the DNA binding of COUP-TFI, because the amount of the receptor interacting with the CRS2 element from the bovine *CTP17* promoter in an electrophoretic mobility shift assay remained constant (data not shown).

The COUP-TFI mutant, COUP-TFIΔ35(1–388) does not contain a repressor domain (the C-terminal 35 amino acids) as shown previously (Leng *et al.* 1996, Shibata *et al.* 1997a, 2003b). Overexpression of Gal4-COUP-TFIΔ35 showed

Figure 5 Ubc9 potentiates basal repression activity of Gal4-COUP-TFI or COUP-TFI in COS-1 cells. (A) COS-1 cells were transfected with 0.43 μ g total DNA including 30 ng Gal4 (lanes 1–4), Gal4-COUP-TFI (lanes 5–11), or Gal4-COUP-TFI Δ 35 cDNA (lanes 12–18), 0.3 μ g 4xGal4-tk-luciferase reporter DNA, and increasing amounts of Ubc9 or mutant expression plasmids (50 and 100 ng) for each well of the 24-well dish as indicated. (B) Experiments similar to those in Fig. 5A were performed, except that Gal4-VP16 was used instead of Gal4-COUP-TFI. COS-1 cells were transfected with 0.41 μ g total DNA including 10 ng Gal4-VP16 cDNA, 0.3 μ g 4xGal4-tk-luciferase reporter DNA, and increasing amounts of the Ubc9 expression plasmids (50 and 100 ng) for each well of the 24-well dish as indicated. (C) Reversal of COUP-TFI-mediated squelching of the basal repression activity of Gal4-COUP-TFI by Ubc9. COS-1 cells were transfected with 0.7 μ g total DNA including 30 ng Gal4 or Gal4-COUP-TFI cDNA, 0.3 μ g 4xGal4-tk-luciferase reporter DNA, 0.27 μ g COUP-TFI cDNA, and increasing amounts of the Ubc9 expression plasmids (50 and 100 ng) for each well of the 24-well dish as indicated. (D) COS-1 cells were transfected with 0.43 μ g total DNA including 30 ng pRSV or pRSV-COUP-TFI cDNA, 0.3 μ g 3xCRS2-SV40-luciferase reporter DNA (pGL3-3 CRS2), and increasing amounts of Ubc9 or mutant expression plasmids (30 and 100 ng) for each well of the 24-well dish as indicated. Assays were performed in three separate experiments, each with triplicate samples.

no significant effects on Gal4-responsive reporter activities and coexpression of Ubc9 did not repress the Gal4-COUP-TFI Δ 35-mediated activities (lanes 12–18 in Fig. 5A).

As shown previously (Shibata *et al.* 1997a), overexpression of COUP-TFI can squelch the repressor activity of a reporter gene (4 $\times$ Gal4-tk-Luc) mediated by Gal4-COUP-TFI (lane 3 in

Fig. 5C). If Ubc9 is a limiting factor required for COUP-TFI-mediated repression, overexpression of Ubc9 should reverse this squelching activity. Overexpression of wild-type Ubc9 or Ubc9(C93S), relieved the squelching of COUP-TFI on Gal4–COUP-TFI repression function in a dose-dependent manner (lanes 4–7 in Fig. 5C), but mutant Ubc9(1–58) did not (lanes 8–9 in Fig. 5C). We also examined the effect of Ubc9 on another COUP-TF-responsive reporter plasmid containing three copies of COUP-TF binding elements from the bovine *CYP17* gene ($3 \times \text{CRS2-SV40-Luc}$) (Bakke & Lund 1995, Shibata *et al.* 2003a,b). The results showed that coexpression of wild-type Ubc9 or mutant Ubc9(C93S) potentiated the repressor function of COUP-TFI in COS-1 cells in a dose-dependent manner (Fig. 5D). However, Ubc9 did not affect the activities of the reporter in the absence of cotransfected COUP-TFI (lanes 1–4 in Fig. 5D). These findings confirm that Ubc9 can function as a corepressor for COUP-TFI-mediated repression activity in mammalian cells.

Ubc9 contains intrinsic repression domains

Corepressors can inhibit the activity of nuclear receptors by several mechanisms. The nuclear receptor corepressors N-CoR and SMRT have been shown to repress the basal activity of promoters when they are recruited as Gal4 DNA binding domain fusion proteins. We therefore tested the intrinsic, transferable repression activity of Ubc9 in this manner and found that the Ubc9–Gal4 DNA binding domain fusion protein repressed the thymidine kinase (tk) promoter with four upstream Gal4 binding sites. Thus Ubc9 has the ability to repress promoters by recruitment alone.

Gal4 DNA binding domain fusions were also generated with Ubc9(1–58), Ubc9(1–75), Ubc9(1–89), Ubc9(1–100), Ubc9(59–158) and Ubc9(76–158) to determine which region(s) retained the intrinsic repression activity of the entire protein (Figs 1, 6A and 6B). Most of these proteins, except Ubc9(90–158) were able to repress the Gal4–tk–Luc reporter (Fig. 6A), indicating that the region encompassing amino acids 1–89 functions as the transcriptional repression domain of Ubc9. In addition, the C93S mutation of Ubc9 fused to the Gal4 DNA binding domain continued to repress the Gal4–tk–Luc reporter activity to a level similar

to that of the wild-type Ubc9, indicating that the SUMO-1 conjugating activity is not required for transcriptional repression activity.

Because it is very important to elucidate the complex of transcription factors that interact with Ubc9 in mammalian cells, we first examined the effect of trichostatin A (TSA), a histone deacetylase inhibitor, on the transcriptional repression activity of Gal4–Ubc9(1–158). The TSA treatment of COS-1 cells at 10^{-7} M for 6–12 h transfected with Gal4 DNA binding domain alone (Gal4) and with Gal4–COUP-TFI significantly reversed their transcriptional activities, whereas TSA treatment at 10^{-10} – 10^{-7} M for 6–12 h did not affect the transcriptional repression mediated by Gal4–Ubc9 (Figs 6B and C). These findings suggest that Ubc9-mediated transcriptional repression probably does not involve recruitment of TSA-sensitive histone deacetylase(s) to the chromatin template. Coimmunoprecipitation experiments and yeast two-hybrid assays showed that Ubc9 is not directly associated with histone deacetylase HDAC1, HDAC3 or mSin3 (data not shown). Transcriptional repression mediated by COUP-TFI involves HDAC-dependent and -independent mechanisms; the latter may be partly mediated by Ubc9.

Tissue distribution of mRNA of Ubc9 and COUP-TFI by northern blot analysis

We next examined the expression of Ubc9 mRNA in human endocrine tissues (Fig. 7). Three Ubc9 mRNAs, 4.4, 2.8 and 1.5 kb, were observed, as shown previously (Wang *et al.* 1996), suggesting that these are derived from alternative splicing of the Ubc9 gene.

Ubc9 mRNA is widely distributed in many endocrine tissues, such as testis, ovary and adrenal cortex, and the expression profile of Ubc9 is thus similar to that of COUP-TFI.

Discussion

In this study, we have identified and described Ubc9, which interacts with the COUP-TFI and other nuclear receptors and has several interesting properties. It has class E2 SUMO-1 conjugating enzyme activity, interacts with COUP-TFI and represses the activity of the COUP-TFs. The C93S substitution of Ubc9, which abrogates the

Figure 6 Autonomous transcriptional repression activity of Ubc9. (A) Autonomous transcriptional repression domains of Ubc9. Luciferase assays of extracts of COS-1 cells transiently transfected with 0.5 μ g total DNA including 0.3 μ g 4xGal4-tk-luciferase reporter and 0.2 μ g expression vectors for Gal4 DBD (pCMV-BD) or several Gal4 DBD-Ubc9 fusion proteins [Ubc9(1-158), (1-58), (1-75), (1-89), (1-100), (59-158), (76-158), (90-158) and (C93S)] for each well of the 24-well dish as indicated. Note that the quantitation shown represents the mean $\pm$ S.D. of luciferase activity in six independent experiments. (B) Luciferase assays of extracts of COS-1 cells transiently transfected with 0.5 μ g total DNA including 0.3 μ g 4xGal4-tk-luciferase reporter plasmid and 0.2 μ g Gal4 DBD or Gal4 DBD-Ubc9 constructs [Ubc9(1-158), (C93S) and (1-58)] for each well of the 24-well dish as indicated. Transfected COS-1 cells were exposed to 10⁻⁷M TSA (■) or DMSO (□), as indicated, for 12 h before harvesting and the luciferase assays. (C) Luciferase assays of extracts of COS-1 cells transiently transfected with 0.5 μ g total DNA including 0.3 μ g 4xGal4-tk-luciferase reporter plasmid and 0.2 μ g Gal4, Gal4-COUP-TFI or Gal4-Ubc9 constructs for each well of the 24-well dish as indicated. Transfected COS-1 cells were exposed to 10⁻¹⁰–10⁻⁷ M TSA or DMSO (□), as indicated, for 6 (■) or 12 h (▨) before harvesting and the luciferase assays.

SUMO-1 conjugating activity, continued to interact with COUP-TFs and to potentiate transcriptional repression mediated by COUP-TFI, indicating that its SUMO-1 conjugase and corepressor activities represent distinct and separable functions.

Ubc9 meets all the criteria for a transcriptional intermediary protein in the modulation of COUP-TFI transcriptional properties. Firstly, as shown in Figs 2–4, Ubc9 specifically interacted with COUP-TFI in yeast, solutions and mammalian cells. The putative ligand-binding domain of COUP-TFI is described as a transcriptional repressor domain

(Leng *et al.* 1996, Shibata *et al.* 1997a). In addition, Ubc9 was shown to interact with the region of COUP-TFI encoding amino acids 388–403, indicating that the corepressor role of Ubc9 is compatible with the properties of interaction with COUP-TFI. Secondly, overexpression of Ubc9 had no effect on the reporter activities mediated by the Gal4 DNA binding domain alone or by Gal4-VP16 in COUP-TFI-negative COS-1 cells. However, Ubc9 potentiated the repression activity mediated by Gal4-COUP-TFI. Subsequently, coexpression of the Ubc9 deletion mutant encoding amino acids 1–58, which impairs COUP-TFI

Figure 7 Tissue distribution of Ubc9 and COUP-TFI mRNA demonstrated by northern blot analyses. A human tissue northern blot (Clontech) was probed with the 1.1 kb hUbc9 cDNA. Each lane contained 2 μ g poly(A)⁺ RNA from pancreas, adrenal medulla, thyroid, adrenal cortex, testis, thymus, small intestine, stomach (lanes 1–8), spleen, thymus, prostate, testis, ovary, small intestine, colon and peripheral leucocytes (lanes 9–16). The positions of RNAs of various sizes are indicated by arrows. COUP-TFI or GAPDH cDNA was used to probe the same blot.

binding, did not affect the repressor activity mediated by Gal4–COUP-TFI, indicating that interaction of COUP-TFI with Ubc9 is required for COUP-TFI-mediated repression activity. The effect of Ubc9 on gene transcription was also confirmed in luciferase assays by using another reporter containing 3 $\times$ CRS2 sequences, to which COUP-TF can bind (Bakke & Lund 1995, Shibata *et al.* 2000a,b) (Fig. 5D). Thirdly, Ubc9 contained an intrinsic repressor domain when fused to the Gal4 DNA binding domain in mammalian cells. As shown in Fig. 6A, most Ubc9 fragments except the one encoding amino acids 90–158 showed intrinsic repressor activity in a dose-dependent manner, suggesting that the N-terminal Ubc9 encoding amino acids 1–89 contains transferable autonomous repressor domain. Therefore, Ubc9 is able to repress promoter activity by recruitment alone. Fourthly, overexpression of Ubc9 relieved the squelching effects of COUP-TFI on Gal4–COUP-TFI repressor function, as shown in Fig. 5C, suggesting that Ubc9 is a limiting endogenous corepressor required for COUP-TFI-mediated

repression. Therefore, Ubc9 possesses all the characteristics expected for a transcriptional corepressor protein of COUP-TFs *in vivo*.

To confirm further that Ubc9 is a corepressor of COUP-TFI, we ruled out several other possible ways in which Ubc9 might enhance COUP-TFI-mediated repression activity. Firstly, as SUMO-1 conjugation by Ubc9 plays an important part in protein modification, the effect of Ubc9 on COUP-TF repression activity might be the result of an effect of Ubc9 on COUP-TFI protein concentrations. Our preliminary results showed that overexpression of Ubc9 did not alter COUP-TFI protein concentrations in COS-1 cells (data not shown). Secondly, it was also possible that overexpression of Ubc9 increases the concentrations of some corepressors or decreases the concentrations of some coactivators, which have been shown to interact with COUP-TFI (Pipaon *et al.* 1999, Sugiyama *et al.* 2000), but the results showed that overexpression of Ubc9 did not alter the protein concentrations of SMRT, SRC-1 and GRIP-1 in the cells (data not shown). As these

experiments were performed by transient transfection, we are not able to conclude unequivocally that Ubc9 has no effects on these protein concentrations, and further investigation are certainly required. Thirdly, another possibility is that overexpression of Ubc9 increased the DNA-binding affinity of COUP-TFI. To exclude this possibility, we carried out electrophoretic mobility shift assays to determine whether or not bacterially or *in vitro* transcription-translated Ubc9 protein affects the binding of COUP-TFI to its response element DNA (CRS2) of the bovine *CYP17* promoter region. The results indicated that Ubc9 has no effects on COUP-TFI binding to the CRS2 element. Taken together with the above mentioned data, these findings indicate that Ubc9 clearly functions as a novel corepressor of COUP-TFI *in vivo*.

Although COUP-TFI has been shown to interact directly with TFIIB (Tsai *et al.* 1987, Ing *et al.* 1992) *in vitro*, we (Shibata *et al.* 1997a) and others (Bailey *et al.* 1997) have previously shown that both N-CoR and SMRT are capable of acting as corepressors for COUP-TFI-mediated gene silencing. A number of COUP-TF-interacting proteins have recently been identified by means of a yeast two-hybrid system. In addition to N-CoR and SMRT, the COUP-TFII ligand-binding domain binds Alien (Dressel *et al.* 1999) and the N-CoR variant RIP13Δ1 corepressor proteins (Bailey *et al.* 1997). In the brain, COUP-TFI is coexpressed with the zinc finger proteins CTIP1 and CTIP2 (Avram *et al.* 2000), which bind COUP-TFI and act as corepressor proteins. Friend of GATA2 (FOG-2) (Huggins *et al.* 2001) has also been shown to interact selectively with the ligand-binding domain of COUP-TFI and COUP-TFII. Thus selective use of tissue-restricted corepressor proteins may be a mechanism by which COUP-TFs can induce tissue-specific transcription.

Ubc9 has several distinct properties that differ from those of the N-CoR and SMRT corepressors. Both N-CoR and SMRT have been shown to interact with both unliganded TRβ and COUP-TFs (Shibata *et al.* 1997a). Conversely, Ubc9 has been shown to interact with COUP-TFs and SF-1, in addition to agonist-bound androgen receptor (Poukka *et al.* 1999, 2000) and glucocorticoid receptor (Gottlicher *et al.* 1996, Kaul *et al.* 2002), but not unliganded TRβ. Therefore, the receptor selectivities of N-CoR/SMRT and Ubc9 differ considerably.

Transcriptional repression mediated by COUP-TFI is enhanced by N-CoR/SMRT and by Ubc9, but the mechanisms appear to be different. The acetylation status of chromatin associated with particular genes is dependent on the activity of both histone acetyltransferases (HATs) and HDACs. The latter are primarily involved in repression of gene transcription by virtue of the compaction of chromatin structure that accompanies the removal of charge-neutralizing acetyl groups from the histone lysine tails. The next question was how Ubc9 potentiates COUP-TFI-mediated transcriptional repression; to answer it, TSA, a specific inhibitor of HDAC, was added in the same transfection assay (Shibata *et al.* 2003b, Marks *et al.* 2001, Hermanson *et al.* 2002, McKenna & O'Malley 2002). TSA treatment was shown to reverse repression by Gal4 and Gal4-COUP-TFI, but not by Gal4-Ubc9 (Figs 6B and C). These results suggest that Ubc9 exerts its repressive effects on target gene promoters by a mechanism distinct from those previously described for nuclear hormone receptors, such as TR or RAR. As COUP-TF has been shown to associate with HDACs through its C-terminal repression domain (Smirnov *et al.* 2000), both HDAC-dependent and HDAC-independent mechanisms have crucial roles in the transcriptional repression mediated by COUP-TFI, the latter being partly mediated by recruitment of Ubc9. As shown in Fig. 6C, Gal4-COUP-TFI-mediated transcription is still repressed by approximately 50% as compared with Gal4-mediated transcription; therefore we presume that COUP-TFI-mediated transcriptional repression function is exerted through TSA-sensitive HDAC complexes including N-CoR and SMRT, in addition to TSA-insensitive HDAC complexes, including Ubc9 complexes. Nevertheless, detailed mechanisms remain to be clarified in future studies. There is also another example of a COUP-TF corepressor protein, CTIP1/Evi9/B cell leukaemia 11a (Avram *et al.* 2000), which has a repression function that appears to be independent of TSA-sensitive HDACs. These findings suggest that alternative mechanisms, perhaps recruitment of members of the TSA-insensitive and NAD⁺-dependent HDACs of the silent information regulator 2 (Sir2) family (Imai *et al.* 2000) or association with heterochromatin, or both, may underlie the corepression mechanisms.

In contrast, the transcriptional properties of Ubc9 have some similarities to those of N-CoR and SMRT, namely bifunctional coactivator and corepressor functions. Ubc9 has been shown to act as a coactivator-like protein of androgen receptor (Poukka *et al.* 1999, 2000) and glucocorticoid receptor in a hormone-sensitive manner (Gottlicher *et al.* 1996, Kaul *et al.* 2002), and thus Ubc9 appears to be a bifunctional coregulator with both coactivator and corepressor functions, depending on the receptor and the promoter contexts. Similarly, SMRT previously identified as a corepressor is shown to function as a coactivator for TR from a negative hormone response element. The mechanisms for the bifunctional role are unknown; however, the role of the allosteric effects of DNA on the transcription factors that bind to it has been reported. Three other examples of bifunctional coregulators, NSD1 (Huang *et al.* 1998), FKHR (Zhao *et al.* 2001) and Zac1b (Huang & Stallcup 2000), have been reported which can regulate transcription either positively or negatively, depending on both the promoter context and the cell type.

Attachment of SUMO is a dynamic and reversible process, requiring a multi-step reaction catalysed by specific enzymes related to, but distinct from, enzymes involved in ubiquitination. The specific SUMO-1 conjugating enzyme is called Ubc9 and is necessary for protein substrate recognition and SUMO-1 linkage, which explains why numerous Ubc9-interacting proteins proved to be substrates for SUMO-1. However, a large number of proteins shown to interact with Ubc9 in the yeast two-hybrid system have not been found to be SUMOylated.

The next question was whether SUMOylation is related to corepressor function. As SUMO-1 is transferred at the Cys93 residue by a class E1 enzyme and replacement of the residue disrupts SUMO-1 binding to Ubc9, introduction of the C93S substitution into the protein-coding sequence of Ubc9 rendered it incapable of SUMO-1 conjugation (Chakrabarti *et al.* 1999, Poukka *et al.* 1999, 2000). The C93S mutant of Ubc9 only marginally attenuated the interaction with COUP-TFI (Fig. 2B), COUP-TFII and SF-1 (data not shown), and thus the SUMO-1 conjugating ability of Ubc9 is not required for interaction with COUP-TFI. In addition, the C93S mutant of Ubc9 continued to potentiate repression activity

mediated by COUP-TFI and Gal4-COUP-TFI, to almost the same extent as wild-type Ubc9. These findings suggest that Ubc9 can function as a corepressor for COUP-TF in a manner that is independent of its SUMOylation activity. However, these findings do not exclude a possibility that COUP-TFI can be SUMOylated by Ubc9 and class E3 SUMO-1 ligase PIAS1. There are no SUMOylation consensus motifs in the human COUP-TFI and our preliminary data indicated that COUP-TFI was not SUMOylated *in vitro* and *in vivo*, but details of the relationship between SUMOylation and transcriptional corepressor activities remain to be elucidated.

Recent data have raised the intriguing possibility that SUMO modification may have a specific impact on the ability of some transcription factors to function synergistically (Iniguez-Lluhi & Pearce 2000, Le Drean *et al.* 2002, Verger *et al.* 2003). Previous studies of the glucocorticoid receptor had identified a region referred to as a synergy control (SC) motif, mutation of which led to a selective increase in the activity of the glucocorticoid receptor from promoters bearing multiple, but not single, sites. The SC motif contains a consensus SUMO acceptor site, and recent data have shown that this is, in fact, the major site of addition of SUMO in the glucocorticoid receptor. It was originally proposed that the presence of multiple SC motifs (now known to encompass SUMOylation sites) allows for the recruitment of a putative SC factor that attenuates synergistic activation (Iniguez-Lluhi & Pearce 2000, Le Drean *et al.* 2002). Recent findings suggest that the negative factor is likely to be SUMO, or potentially the E2 conjugase Ubc9, which is capable of binding SUMO consensus sites. As there are no SUMO consensus sites in COUP-TFI, and overexpression of SUMO-1 did not affect the COUP-TFI-mediated transcriptional activity (data not shown), the role of SUMO-1 for COUP-TFI remains unknown. Similarly, the SUMO E2 enzyme Ubc9, which interacts with the androgen receptor and with the transcriptional repressor TEL, seems to regulate their activities independently of its SUMO E2 enzyme activity. It is possible that proteins associated with COUP-TFI may be targeted by SUMOylation; these SUMO substrates need to be identified. Indeed, there are SUMO consensus sites in several HDACs, and SUMOylation is crucial for the expression of HDAC activities (David *et al.* 2002, Kirsh *et al.* 2002).

Ubc9 has been shown to be essential both for cell viability and for SUMO-1 conjugase. Hayashi *et al.* (2002) showed that a considerable portion of the Ubc9-depleted cell population was committed to apoptosis without accumulating in a specific phase of the cell cycle, suggesting that chromosome damage accumulates in Ubc9-depleted cells.

Ubc9 is highly expressed in steroidogenic tissues such as the adrenal cortex, testis and ovary. In addition, it has been shown to interact not only with COUP-TFs but also with SF-1, indicating that Ubc9 together with COUP-TFs and SF-1 have an important role in steroidogenesis of the adrenal cortex. We found that Ubc9 can function as a corepressor of COUP-TFI-mediated repression of the *CYP17* gene that is a key enzyme for glucocorticoid and adrenal androgen synthesis. We also found that Ubc9 was highly expressed in the nuclei of the normal adrenal cortex, in addition to adrenocortical adenomas (data not shown). We are currently investigating the pathophysiological importance of Ubc9 for COUP-TFI and SF-1 in adrenocortical tumours.

Collectively, our results suggest that Ubc9 functions as a novel COUP-TFI corepressor, the function of which is distinct and separable from its SUMO-1 conjugating enzyme activity. Further investigation is needed to explore the mechanistic role of SUMO-1 in COUP-TFI-mediated transcription.

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