

Wwp2, an E3 Ubiquitin Ligase That Targets Transcription Factor Oct-4 for Ubiquitination*

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The POU transcription factor Oct-4 is a master regulator affecting the fate of pluripotent embryonic stem cells. However, the precise mechanisms by which the activation and expression of Oct-4 are regulated still remain to be elucidated. We describe here a novel murine ubiquitin ligase, Wwp2, that specifically interacts with Oct-4 and promotes its ubiquitination both *in vivo* and *in vitro*. Remarkably, the expression of a catalytically inactive point mutant of Wwp2 abolishes Oct-4 ubiquitination. Moreover, Wwp2 promotes Oct-4 degradation in the presence of overexpressed ubiquitin. The degradation is blocked by treatment with proteasome inhibitor. Fusion of a single ubiquitin to Oct-4 inactivates its transcriptional activity in a heterologous Oct-4-driven reporter system. Furthermore, overexpression of Wwp2 in embryonic stem cells significantly reduces the Oct-4-transcriptional activities. Collectively, we demonstrate for the first time that Oct-4 can be post-translationally modified by ubiquitination and that this modification dramatically suppresses its transcriptional activity. These results reveal that the functional status of Oct-4, in addition to its expression level, dictates its transcriptional activity, and the results open up a new avenue to understand how Oct-4 defines the fate of embryonic stem cells.

The POU transcription factor Oct-4 (also called Oct-3, encoded by Pou5f1) is known to be critical in mammalian embryonic development (1–4). It is expressed almost exclusively in totipotent and pluripotent cells during mouse development and also is present in cultured undifferentiated embryo cell lines including embryonic stem (ES)¹ cells, embryonal carcinoma cells, and embryonic germ cells. However, Oct-4 is absent from

all of the differentiated somatic cell types *in vitro* and *in vivo* (5, 6), suggesting its important role in maintaining cellular pluripotency. The deletion of Oct-4 in mice causes an early lethality at 3.5 days of gestation. The inner cell mass cells are not pluripotent. Instead, cells at the morula stage differentiate into a trophoblastic lineage (7). Thus, Oct-4 has an essential function in the establishment of the pluripotential inner cell mass lineage in preimplantation development. Further investigation via conditional repression and expression of Oct-4 in ES cells demonstrates that the level of Oct-4 expression governs the developmental fate of ES cells (8). Therefore, Oct-4 transcription factor can be considered as a master regulator for initiation, maintenance, and differentiation of pluripotent cells (9). There has to be tight regulation of Oct-4 expression and activity for it to maintain the pluripotent state of ES cell. However, it is not known how such tight regulation is realized *in vivo*.

A large number of recent findings have highlighted the close relationship between transcription regulation and the ubiquitin (Ub)-proteasome pathway (10, 11). The best-studied function of ubiquitination is its role in protein degradation where polyubiquitinated proteins are recognized by the 26 S proteasome and are degraded rapidly (12, 13). However, other functions for Ub are being discovered at a rapid rate (14–16). Ubiquitination is regulated by a cascade of enzymatic reactions resulting in the covalent addition of Ub, a 76 amino acid polypeptide, to target proteins. Ub is first activated by the E1 Ub-activating enzyme in an ATP-dependent reaction, resulting in thioester bond formation between a specific cysteine of the enzyme and the carboxyl terminus of Ub. The activated Ub is then transferred to one of many different E2s (Ub-conjugating enzyme or Ub-carrier enzyme). E2 enzymes then mediate the transfer of Ub to the target protein directly or to E3 Ub protein ligases, which are responsible for substrate recognition and for promoting Ub ligation to the ϵ -amino group of lysine residues on a substrate. The E3 ligases are critical components that are needed to determine the enzymatic specificity in the Ub cascade as a result of direct interaction with substrates (13). Two distinct E3 families have now been identified. One family of E3 ligases is RING finger E3, which is believed to mediate the direct transfer of Ub from E2 to substrates (13, 17). Members of the other family of E3 Ub ligases accept activated Ub from E2 and form a thioester intermediate with Ub. The first identified member of the latter family is E6-associated protein, which mediates polyubiquitination of p53 (18, 19). Other members of this family include Nedd4 and related Nedd4-like proteins (20). These all have a carboxyl-terminal domain termed HECT (for homologous to E6-AP carboxyl terminus) domain, which provides the Ub ligase enzyme activity. E3 ligases of Nedd4 family

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¹ The abbreviations used are: ES, embryonic stem; Ub, ubiquitin; E1, ubiquitin-activating enzyme; E2, ubiquitin carrier protein; E3, ubiquitin-protein isopeptide ligase; HECT, homologous to E6-AP carboxyl terminus; GST, glutathione S-transferase; DIG, digitoxin; HEK, human embryonic kidney; CA, cysteine to alanine; STAT, signal transducers and activators of transcription.

contain 2–4 tryptophan-based WW domains, which consist of 35–40 amino acids and bind certain proteins containing proline-rich motifs. The WW domains have been reported in a wide variety of proteins of yeast, nematode, vertebrate, and mammalian origins and play a direct role in mediating specific and distinct protein-protein interactions (21–23).

We describe here a murine E3 Ub ligase, Wwp2, that can directly bind to Oct-4. This interaction occurs through WW domains of Wwp2. Wwp2 promotes ubiquitination of Oct-4 both *in vitro* and *in vivo*. The enzymatic activity of Wwp2 is dependent on the integrity of its HECT domain. Furthermore, overexpression of Wwp2 in embryonic stem cells significantly suppresses trans-activation activity of Oct-4. Importantly, Wwp2 expression is decreased in parallel with the decrease in Oct-4 expression when ES cells are induced into differentiation. These results suggest that Wwp2 may serve as a regulator of Oct-4-mediated functions in ES cells and open up a new avenue to understand how self-renewal and pluripotency are maintained in ES cells.

EXPERIMENTAL PROCEDURES

Construction of Vectors—The cDNA sequences corresponding to the full-length proteins of Oct-4 and Wwp2 were amplified by reverse transcriptase-PCR from 128 and D3 mouse ES cell lines (ATCC), respectively, and cloned into pGEM-T-Easy vector (Promega). The sequences were then subcloned either into pET-30a(+) (Novagen) and pGEX-4T-1 (Amersham Biosciences) vectors for expression in bacteria or pCMV-Not, pCB6-Not (kind gifts from R. Baer) and pcDNA3 (Invitrogen) vectors for expression in mammalian cells. Amino acid residual mutants were generated by PCR-based site-directed mutagenesis. Mouse Itch cDNA was a kind gift from N. G. Copeland. Hemagglutinin-Ub expression vector was a kind gift from D. Bohmann. To generate Ub-Oct-4 fusion protein expression vector, the cDNA sequence of full-length Ub (GenBank™ accession number AK080925) was amplified by reverse transcriptase-PCR from the D3 mouse ES cell line. For expression of Wwp2 and Oct-4 in CGR8 ES cells (a kind gift from A. Smith), the cDNA sequences were cloned into a modified X-pPNT vector (24).

Preparation of Nuclear Extracts—The nuclear extract from F9 cells was prepared as described previously (25, 26) and dialyzed against BC200N (20 mM HEPES-NaOH, pH 7.9 at 25 °C, 20% (v/v) glycerol, 200 mM KCl, 0.2 mM EDTA, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, and 0.1% Nonidet P-40).

Fusion Protein Expression and GST Purification—GST and His fusion proteins were expressed and prepared according to the manufacturer's instructions from Amersham Biosciences and Novagen, respectively. GST or GST-Oct-4N (the amino-terminal residues 29–124 of Oct-4) proteins bound to glutathione immobilized on Sepharose beads (Amersham Biosciences) in equal molar amounts were incubated overnight at 4 °C with 2–4 mg of the nuclear extracts prepared as described above. The bound proteins and beads were washed and then eluted with 250 μ l of glutathione elution buffer (Amersham Biosciences). The samples were suspended in SDS-PAGE loading buffer and applied to a 10% SDS-PAGE gel. Following electrophoresis, the gel was stained with Coomassie Blue. The bands only present in GST-Oct-4N lane were cut off for mass spectrometry analysis.

In-gel Digestion of Proteins and Capillary-High Pressure Liquid Chromatography Mass Spectrometric Analysis for Protein Identification—The protein in-gel digestion and protein identification by nano-high pressure liquid chromatography/mass spectrometry/mass spectrometry was carried out according to the procedures described previously (27).

Northern and Western Blot Analyses—A mouse multiple tissue Northern blot (KINGRACE) was hybridized with digitoxin (DIG)-labeled cDNA probes of Wwp2 (682 bp of 302–983 of NM_025830.3). The equivalent loading of mRNA in each lane was confirmed by hybridization of the same blot with a DIG-labeled β -actin cDNA probe. Hybridization and detection were performed with a DIG-labeling and detection kit (Roche Applied Science). For Western blot analysis, cells were lysed as described previously (28). The protein concentration of each supernatant was determined by the Bradford method (29). For experiments involving samples from transiently transfected cells, the cotransfected pSV- β -galactosidase plasmid (a kind gift from R. Baer) was used to normalize each sample. Western analysis was conducted by enhanced chemiluminescence (Pierce). The Western blot analysis was performed in at least three different experiments, and representative data are shown.

Antibodies—Antibodies against Oct-4 and Wwp2 were raised in rabbits against the purified GST-Oct-4N fusion protein and GST-Wwp2 M (amino acid residues 140–274 of Wwp2), respectively. The rabbit polyclonal antisera were purified by sequential affinity chromatography on His-Oct-4N or His-Wwp2 M conjugated to CNBr-activated-Sepharose 4B (Amersham Biosciences) and HiTrap protein A-Sepharose (Amersham Biosciences). Monoclonal antibodies against the FLAG epitope (Sigma), the His epitope (Santa Cruz Biotechnology), and the Ub epitope (Cell Signaling) were used for immunoprecipitation and immunoblotting.

Cell Culture and DNA Transfection—HEK 293 cells (a kind gift from R. Baer) were cultured under standard condition and transfected with the calcium phosphate method. F9 cells were grown as suggested by ATCC. Mouse ES cell lines, CGR8, D3, and SS1 (a mouse ES cell line derived in our laboratory with standard method), were cultured as described previously (30). CGR8 cells were transfected with LipofectAMINE™ 2000 (Invitrogen).

Immunoprecipitation and Pull-down Assay—For immunoprecipitation, cell lysates were prepared in a low stringency buffer (10 mM Hepes, pH 7.6, 250 mM NaCl, 0.1% Nonidet P-40, 5 mM EDTA, 1 mM NaF, 5 μ g/ml aprotinin, 1 μ g/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride) and incubated with a specific antibody for 2 h at 4 °C followed by the addition of protein A-Sepharose beads for another 1 h. The coimmunoprecipitation experiments of endogenous Oct-4 and Wwp2 from nuclear extract of F9 cells were performed as described previously (31) with minor modifications. For GST pull-down experiments, 0.5 μ g of GST fusion proteins were incubated with 0.5 μ g of His fusion proteins in 250 μ l of TBS-N (20 mM Tris-HCl, pH 7.6, 200 mM NaCl, and 0.1% Nonidet P-40) at 4 °C for 2 h followed by the addition of glutathione-Sepharose 4B beads for another hour. The samples from immunoprecipitation or GST pull-down assays were analyzed by Western blot.

Ubiquitination Assay in Vivo and in Vitro—For the assay *in vivo*, HEK 293 cells were transfected with 2 μ g of pCMV-Oct-4, 2 μ g of pCMV-FLAG-Wwp2, 4 μ g of pMT 107 (vector for His₆-Ub expression, a kind gift from D. Bohmann), and 2 μ g of pSV- β -galactosidase. 36–48 h after transfection, cells were treated for 5 h with proteasome inhibitor (20 μ M MG132 or Me₂SO) before harvest and then were lysed in Nonidet P-40 lysis buffer (10 mM Tris-HCl, pH 8.0, 1% Nonidet P-40, 100 mM NaH₂PO₄, and 500 mM NaCl). After β -galactosidase activity was measured, the cell lysates were normalized and adjusted to a final concentration of 8 M urea and 10 mM imidazole. The ubiquitinated proteins were affinity-purified on nitrilotriacetic acid His-bind resin (Novagen), resolved by SDS-PAGE, and analyzed by Western blotting with anti-Oct-4 antibody.

In vitro ubiquitination assay was carried out by adding GST-Oct-4 (1 μ g), rabbit E1 (50 ng, Calbiochem), His-Ub-carrier enzyme H7/6 (0.4 μ g, Calbiochem), His-Ub (2 μ g, Calbiochem), and GST-Wwp2 (0.5 μ g) to ubiquitination buffer (50 mM Tris-HCl, pH 7.4, 2 mM ATP, 5 mM MgCl₂, and 2 mM dithiothreitol) to a final volume of 30 μ l. The reactions were incubated at 30 °C for 120 min and stopped by boiling in SDS loading buffer containing 2% SDS and 5% β -mercaptoethanol. Ubiquitinated proteins were visualized by immunoblotting with antibody against Ub or Oct-4.

Luciferase Reporter Assays—HEK 293 or CGR8 ES cells (1×10^6 cells in a 60-mm dish) were transiently transfected with vectors as indicated in the figures. Luciferase activities in the samples were measured using a luciferase reporter assay system (Promega). The activity was normalized by β -galactosidase activity in each sample. The experiments were performed at least three times in duplicate setting. The data are shown as the mean \pm S.D.

Immunofluorescence Staining—Mouse ES cell lines, SS1, were seeded onto microscope slides with feeder cells grown in a 100-mm culture dish. Immunofluorescence staining was performed as described previously (28). The stained cells were mounted under coverslips, and immunofluorescence was recorded using a confocal microscope (Leica TCS SP2) and analyzed by Leica confocal software (TCS SP2, version 2.5.1104).

RESULTS

Identification of Wwp2 as a Novel Murine Oct-4-interacting Protein—To identify Oct-4-associated proteins, we purified Oct-4-interacting proteins from F9-embryonal carcinoma cell line nuclear extract using GST-Oct-4N (residual 29–124 of Oct-4) affinity chromatography. One protein (~100 kDa) was found to be GST-Oct-4N-specific and therefore was excised for mass spectrometric analysis (Fig. 1A). Mass spectrometric

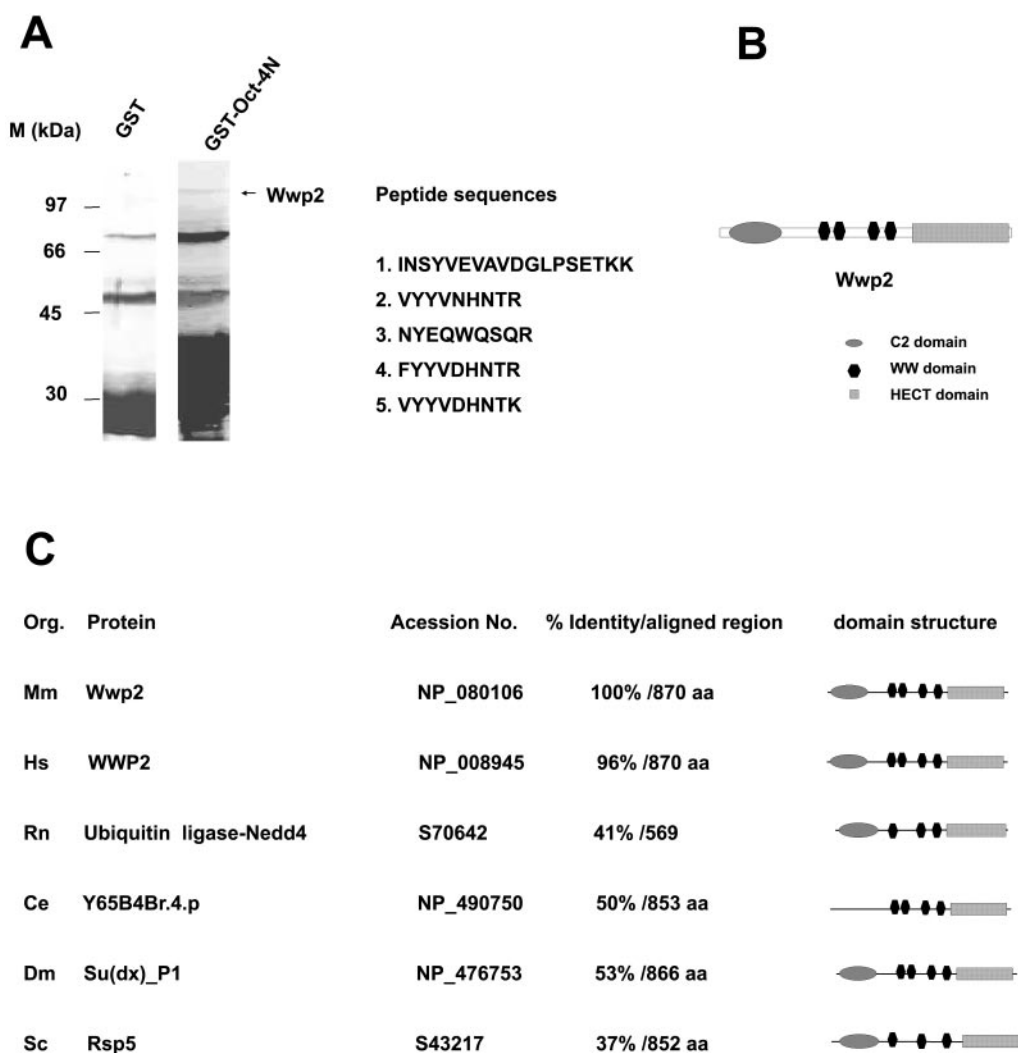


FIG. 1. **Identification of Wwp2.** *A*, purification of Wwp2 from F9 cells. Coomassie Blue staining of SDS-PAGE gel containing the proteins from the indicated columns is shown. Peptide sequences derived from the Wwp2 protein band were obtained by mass spectrometry. *B*, schematic representation of the modular architecture of Wwp2-coding sequence. *C*, domain structure of the Wwp2 homologues in different species. *Mm*, *Mus musculus*; *Hs*, *Homo sapiens*; *Rn*, *Rattus norvegicus*; *Ce*, *C. elegans*; *Dm*, *D. melanogaster*; *Sc*, *S. cerevisiae*; *aa*, amino acid.

analysis identified this protein as the product of murine Riken cDNA 1300010006 gene (NP_080106, WW domain-containing protein 2). Researching the data base and performing reverse transcriptase-PCR, we obtained a cDNA clone of 2703 bp from mouse D3 ES cell line. The sequence includes an open reading frame that encodes a protein of 870 amino acid residues with a predicted molecular mass of 98.7 kDa. The predicted protein contains a protein kinase C conserved region 2 (C2 domain) at its amino terminus and a HECT domain at its carboxyl terminus. In addition, there are four WW domains between the C2 and HECT domains (Fig. 1B). The amino acid sequence is 96% identical to a human Nedd-4-like Ub-protein ligase, which is termed WW domain-containing protein 2, WWP2 (GenBankTM accession number NP_008945) (22). Because of the sequence similarity, we concluded that this novel Oct-4-interacting protein is the murine orthologue of human WWP2 and is designated Wwp2. Furthermore, there are sequences similar to Wwp2 in a number of other organisms including rat, *Arabidopsis thaliana*, *Caenorhabditis elegans*, *Drosophila melanogaster*, and *Saccharomyces cerevisiae*. Analysis of the deduced protein from the Wwp2-homologous sequences and domain structures from different species indicates that Wwp2 is an evolutionarily conserved protein (Fig. 1C).

Wwp2 Is a Ubiquitously Expressed Protein with High Expression in Undifferentiated ES Cells—Northern blot analysis of

RNA from various mouse tissues identified two Wwp2 mRNA transcripts with approximate lengths of 3.0 and 4.4 kb, respectively, with the former being the predominant form (Fig. 2A). Among the tissues examined, the mRNA expression level was highest in testis, relatively high in spleen, kidney, and liver, and low in brain, heart, and lung but absent in skeletal muscle. To examine the intracellular distribution of endogenous Wwp2, immunofluorescent staining of SS1 mouse ES cells was performed with Wwp2 antibody. As shown in Fig. 2B, Wwp2 protein was present both in the nuclei and cytoplasm of the cells (Fig. 2B, *a-c*). In control, Wwp2 protein was not detected in rabbit control IgG-stained cells (Fig. 2B, *d-f*). The same results were also obtained when CGR8 and D3 ES cells were stained (data not shown). The results demonstrate that Wwp2 is a ubiquitously expressed protein and exists in both cytoplasmic and nuclear compartments in ES cells.

To examine whether expression of Wwp2 in ES cell is related to the differentiation status of the cells, CGR8 ES cells were treated with 0.1 μ M retinoic acid and the protein levels of Wwp2 and Oct-4 were determined by Western blot. Apparently, protein levels of both Wwp2 and Oct-4 were decreased when the cells were induced into differentiation (Fig. 2, *C* and *D*). The decrease pattern of Wwp2 was paralleled with that of Oct-4, although the decrease in Oct-4 protein level (<4% left by day 4) was more dramatic than in Wwp2 (~36% left by day 4). The

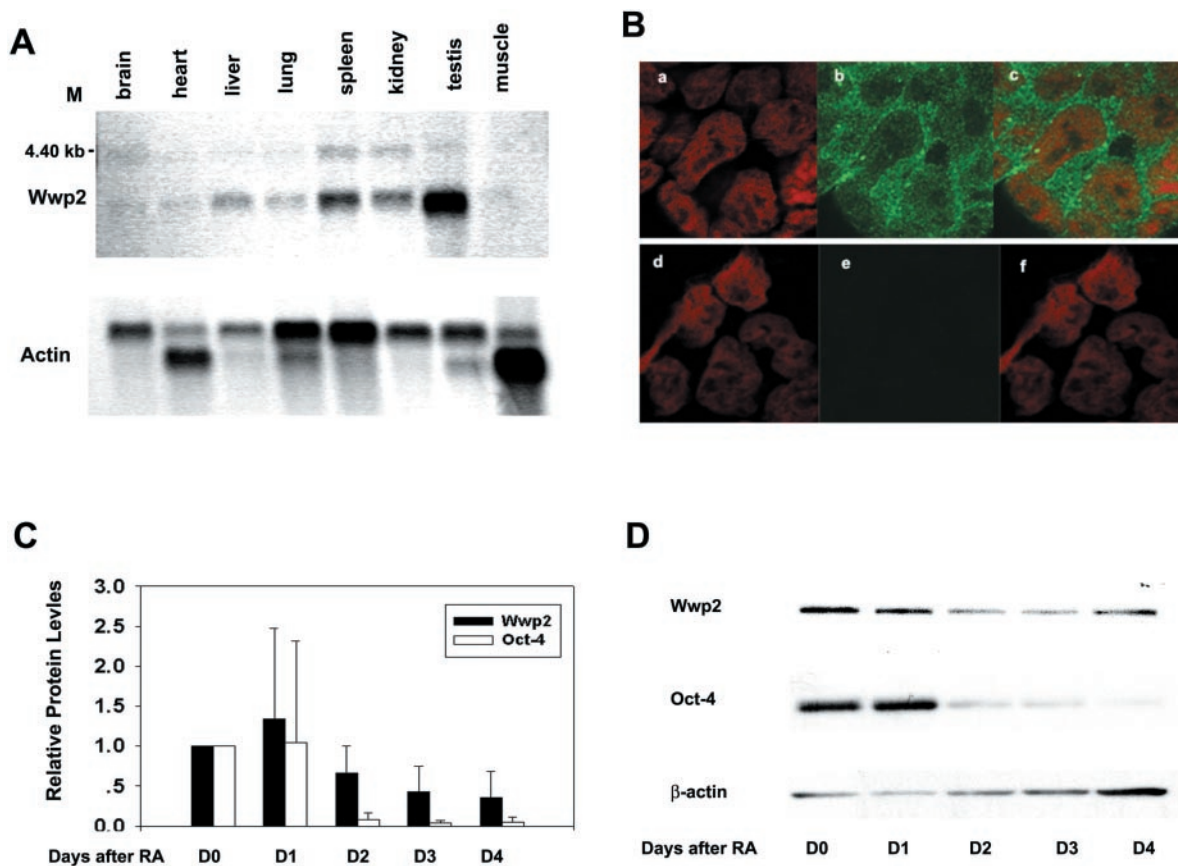


FIG. 2. Expression of Wwp2 in mouse tissues and ES cells. *A*, Northern blot analysis of Wwp2 mRNA expression in mouse tissues with DIG-labeled cDNA probe for Wwp2 (upper panel) and β -actin (lower panel). *B*, Wwp2 exists in both nuclei and cytoplasm of mouse ES cells. SS1 ES cells were stained with propidium iodide to highlight the nucleus (red, *a* and *d*) and with either the Wwp2 antibody (green, *b*) or rabbit IgG from Jackson Laboratories (*e*). *c* is a composite image of *a* and *b*, and *f* is a composite of *d* and *e*. *C*, protein level of Wwp2 in ES cells declines as the cells are induced into differentiation by retinoic acid (RA). CGR8 ES cells were treated with $0.1 \mu\text{M}$ RA and were collected at the indicated times. The protein levels of Wwp2 and Oct-4 in whole cell lysates were determined by Western blot with antibodies against Oct-4 and Wwp2, respectively. The density of specific bands from the Western blot was measured by densitometer and normalized by the density of β -actin. *D*, the Western blot from one of experiments described in *C* is shown.

observation suggests that Wwp2 might be involved in the maintenance of Oct-4 activity within a normal range in ES cells.

Wwp2 Associates with Oct-4 in Vitro and in Vivo—To confirm the association between Oct-4 and Wwp2 detected by affinity purification, GST pull-down assay was performed with bacterially expressed GST fusion protein of Wwp2 and His fusion protein of Oct-4. As shown in Fig. 3*A*, immobilized GST-Wwp2, but not GST alone, was able to pull down His-Oct-4. This indicates that Wwp2 interacts with Oct-4 directly *in vitro*. We next determined whether Wwp2 associated with Oct-4 *in vivo*. Coimmunoprecipitation experiments were performed using lysates of HEK 293 cells expressing exogenous Oct-4 and FLAG-tagged Wwp2 or vector alone. As shown in Fig. 3*B*, Oct-4 coimmunoprecipitated with FLAG-tagged Wwp2. As a control, Oct-4 was not detected in the immunoprecipitates of cells expressing FLAG-Wwp2 or Oct-4 alone, providing evidence for the specific interaction of these two proteins in mammalian cells. Lastly, we examined whether the interaction between endogenous Oct-4 and Wwp2 occurred (Fig. 3*C*). Anti-Oct-4 antibody, but not the Rabbit IgG control, was able to coimmunoprecipitate Wwp2 from nuclear extracts of F9 cells, demonstrating the existence of endogenous Oct-4-Wwp2 complexes in embryonic pluripotent cells. Therefore, we conclude that there is a specific interaction between Oct-4 and Wwp2 both *in vitro* and *in vivo*.

Mapping of Wwp2-interacting Domains—To determine which domains of Wwp2 interact with Oct-4, C2, WW (includ-

ing all four WW domains), and HECT domains of Wwp2 were expressed as GST fusion proteins. Also, a Wwp2 full-length fusion protein and one with a mutation of residue 838 cysteine to alanine (Wwp2-CA) were used. The expression of these GST fusion proteins is shown in Fig. 4*A*, bottom panel. As shown in Fig. 4*A*, top panel, in addition to full-length Wwp2, WW domains bound to Oct-4 were detected by immunoblotting with anti-His antibody. Furthermore, the mutation of cysteine to alanine in the HECT domain of residue 838 did not affect its association with Oct-4. In contrast, C2 and HECT domains were not capable of binding to Oct-4. Therefore, the interaction with Oct-4 is probably mediated through the WW domains of Wwp2.

Mapping of Oct-4-interacting Domains—The amino-terminal region of Oct-4 is rich in proline and acidic residues, whereas the carboxyl-terminal region is rich in proline, serine, and threonine. To determine the regions of Oct-4 involved in the interaction with Wwp2, full-length Oct-4, amino-terminal region (N-96, residue 29–124), and carboxyl-terminal region (C-70, residue 283–352) were expressed bacterially as GST fusion proteins (Fig. 4*B*, bottom panel). GST pull-down assays with His-Wwp2 were performed. As shown in Fig. 4*B*, top panel, in addition to full-length Oct-4, both amino and carboxyl termini of Oct-4 interacted with Wwp2 directly, even when washed in 500 mM of salt condition. As a negative control, GST fusion with amino-terminal 118 amino acids of Rex-1 (GST-Rex1N) could not pull down His-Wwp2 and GST alone could not either. This result indicates that both the amino-terminal

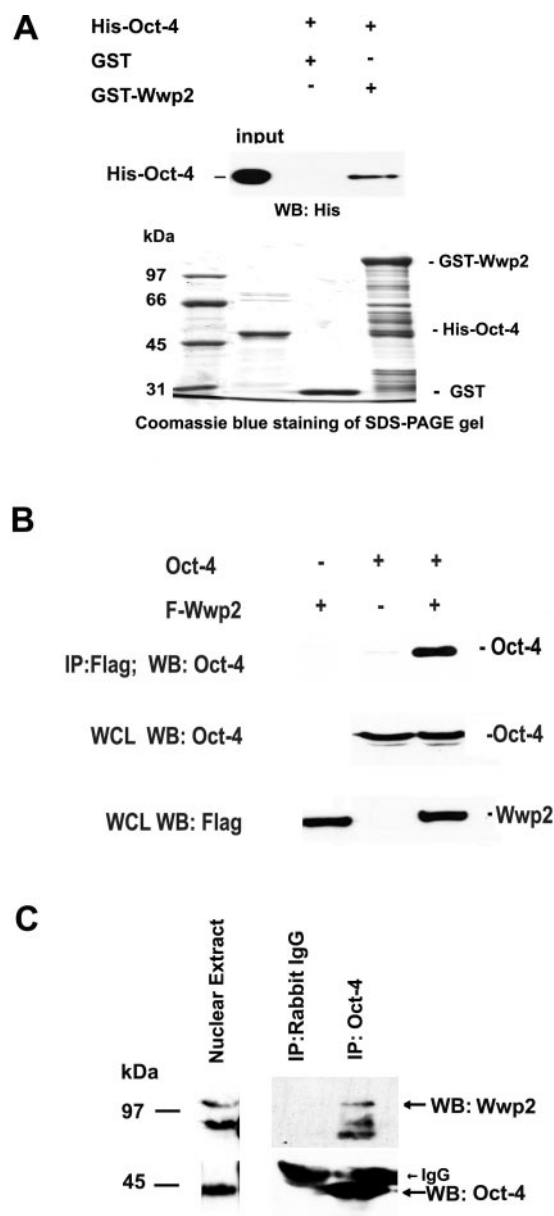


FIG. 3. Wwp2 binds to Oct-4 specifically both *in vitro* and *in vivo*. *A*, bacterially expressed GST-Wwp2 fusion proteins were incubated with His-Oct-4 fusion protein and precipitated by glutathione-Sepharose 4B beads. Associated His-Oct-4 protein was detected by immunoblotting with anti-His-monoclonal antibody (*top panel*). The bacterially expressed His-Oct-4, GST, and GST-Wwp2 fusion proteins were separated on SDS-PAGE gel and stained by Coomassie Blue (*bottom panel*). WB, Western blot. *B*, coimmunoprecipitation of Oct-4 and FLAG-Wwp2 in HEK 293 cells. Constructs encoding Oct-4 and FLAG-tagged Wwp2 were cotransfected into HEK 293 cells. Whole cell lysates (WCL) were immunoprecipitated with anti-FLAG antibody and analyzed by Western blot with anti-Oct-4 antibody (*top panel*). Oct-4 and FLAG-tagged Wwp2 expressed in the cell were confirmed by immunoblotting with anti-Oct-4 or anti-FLAG antibody, respectively (*middle and bottom panels*). *C*, association of endogenous Oct-4 with Wwp2 in F9 cells. The nuclear extract of F9 cells was subjected to immunoprecipitation (IP) with anti-Oct-4 antibody or control rabbit IgG. The immunoprecipitates were analyzed by Western blot (WB) with anti-Wwp2 antibody.

region and carboxyl-terminal region of Oct-4 could interact with Wwp2.

Wwp2 Promotes Oct-4 Ubiquitination—The presence of HECT domain in the carboxyl terminus of Wwp2 suggests that it might be an E3 Ub-protein ligase. To determine whether Wwp2 has an intrinsic E3 activity and whether Oct-4 is indeed a substrate for Wwp2, an *in vitro* ubiquitination assay was

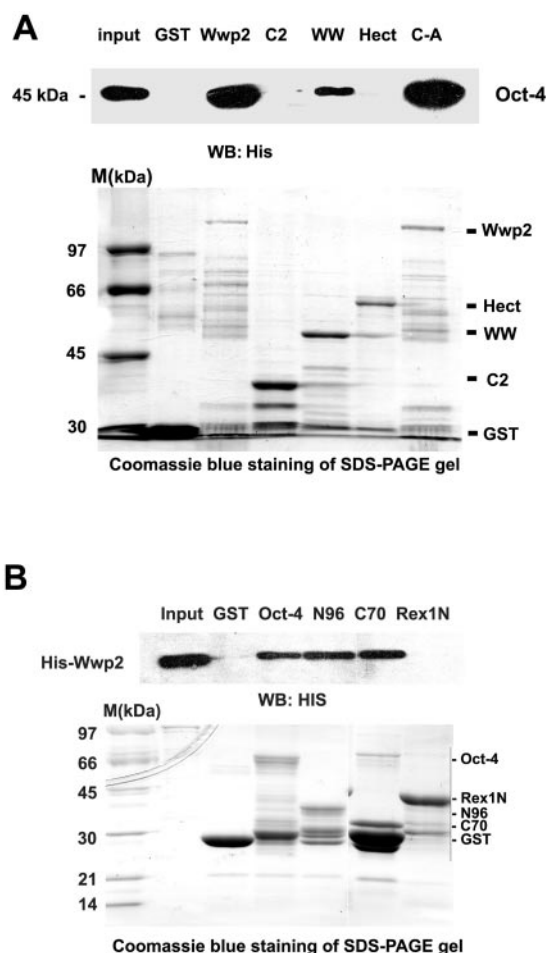


FIG. 4. The interacting regions between Wwp2 and Oct-4. *A*, WW domain of Wwp2 binds to Oct-4. GST fusion proteins of Wwp2 were analyzed by SDS-PAGE and stained by Coomassie Blue (*bottom*). The ability of the various truncated and mutant Wwp2 fusion proteins to bind to His-Oct-4 was analyzed by immunoblotting with antibody against His (*top*). *B*, Oct-4 interacts with Wwp2 through both amino- and carboxyl-activation domains. His-Wwp2 was incubated with GST fusion proteins of Oct-4 or truncated Oct-4. GST and GST-Rex-1N118 were used as negative control. Proteins pulled down by glutathione-Sepharose 4B beads were analyzed by immunoblotting with antibody against His (*top panel*). The various GST fusion proteins were shown by Coomassie Blue-stained SDS-PAGE gel (*bottom*).

performed. As shown in Fig. 5*A*, *left panel*, higher molecular weight species indicative of the addition of Ub moieties to Oct-4 were only seen in the presence of added E1, E2, Ub, and Wwp2 (*lane 5*) and were Oct-4-dependent (not in *lane 6*). A cysteine residue in the HECT domains of E3 ligases of this family is thought to play a crucial role for ubiquitin thioester bond formation and for the ability of E3 to target protein substrates for ubiquitination (18, 32). To examine a possible role of the Wwp2 HECT domain in mediating Oct-4 ubiquitination, the mutant form of Wwp2 (Wwp2-CA) was tested. As expected, Wwp2-CA could not ubiquitinate Oct-4 (*lane 7*), which provides strong evidence for the importance of cysteine at residue 838 in this enzymatic reaction. To further confirm that the ubiquitinated protein is indeed Oct-4, Western blotting was performed with anti-Oct-4 antibody. Again, a higher molecular weight smear was only detected in the presence of E1, E2, Ub, Oct-4, and Wwp2 (*lane 5* in Fig. 5*A*, *right panel*). These observations confirm that Oct-4 can serve as a substrate for Wwp2-dependent ubiquitination *in vitro*.

We next tested whether Wwp2 can mediate Oct-4 ubiquitination *in vivo*. To detect ubiquitinated forms of cellular Oct-4, HEK 293 cells were transfected with expression vectors encod-

A

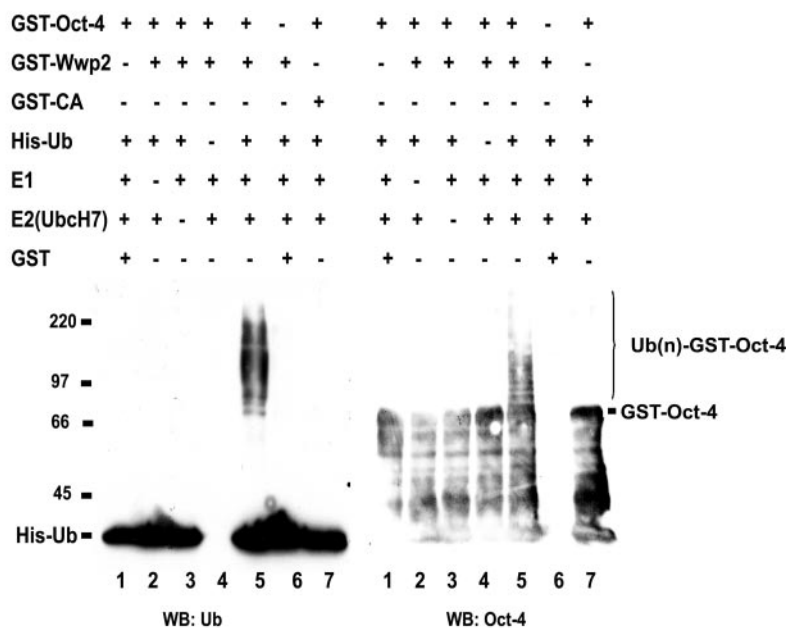
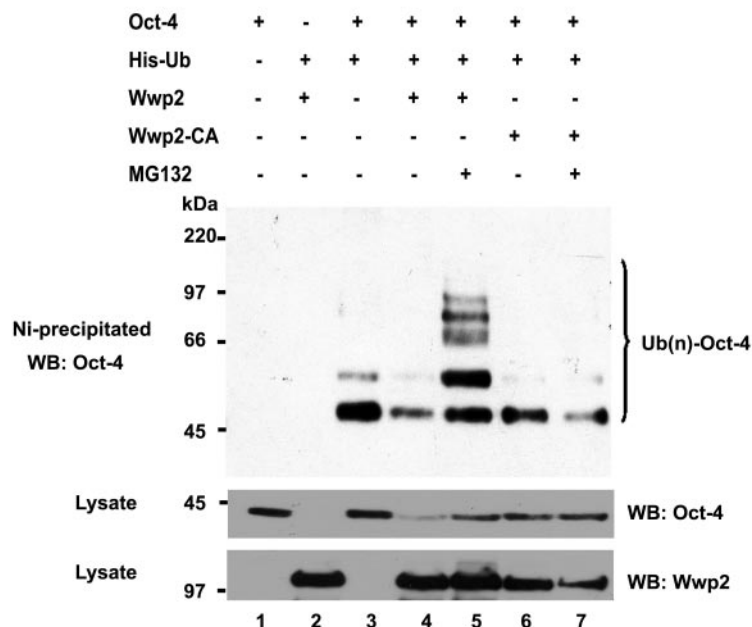


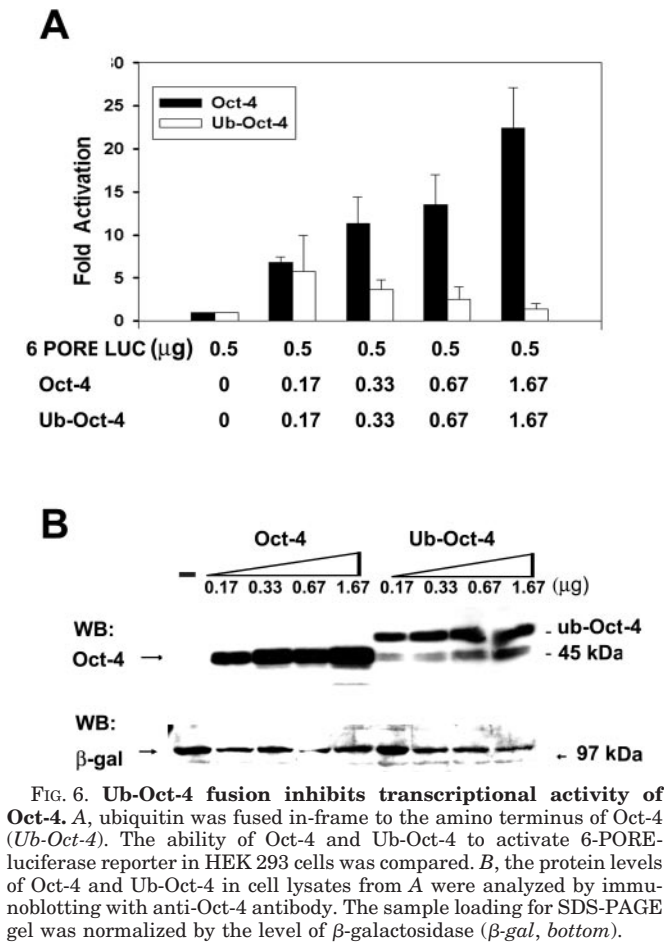
FIG. 5. Wwp2 promotes ubiquitination of Oct-4. *A*, Oct-4 ubiquitination *in vitro* assay. Various purified proteins as indicated were incubated in ubiquitination buffer. Ubiquitinated Oct-4 was visualized by Western blotting (WB) with antibody against Ub (*left panel*) and Oct-4 (*right panel*), respectively. *B*, Wwp2 promotes ubiquitination of Oct-4 in HEK 293 cells. HEK 293 cells were transfected with expression vectors as indicated. MG132 at 20 μ M or Me₂SO was added to cell culture medium 5 h before harvest. Ubiquitinated polypeptides were isolated by nitrilotriacetic acid affinity beads and analyzed by Western blotting with antibody against Oct-4 (*top*). Protein levels of Oct-4 and Wwp2 in the cell lysates were measured by Western blotting with antibodies against Oct-4 (*middle*) and Wwp2 (*bottom*), respectively. Sample loading in each lane was normalized by β -galactosidase activities in each sample. Ni, nickel.

B



ing His-tagged Ub, Oct-4, and Wwp2. Ubiquitinated proteins in the cell lysate were isolated by nitrilotriacetic acid affinity chromatography and then analyzed by immunoblotting with anti-Oct-4 antibody (Fig. 5*B*, *top panel*). Coexpression of His-tagged Ub with Oct-4 caused ubiquitination of exogenously expressed Oct-4 in the higher molecular weight form (*lane 3*). This could be the result of endogenous human WWP2 or other E3 activities present in the cells (the full-length of human WWP2 was cloned from HEK 293 cDNA, data not shown). Surprisingly, the ubiquitinated Oct-4 was reduced instead of being increased when Wwp2 was coexpressed (*lane 4*). Western blot analysis showed that Oct-4 protein level was significantly reduced in the presence of exogenous Wwp2 (*lane 4*, *middle*

panel), which suggests that the reduction in the ubiquitinated Oct-4 might be caused by Wwp2-mediated Oct-4 degradation. To verify this hypothesis, the cells were treated with proteasome inhibitor (MG132) before harvest. Strikingly, ubiquitination of Oct-4 was significantly enhanced by the treatment with MG132 (compare *lane 4* with *lane 5*). Meanwhile, the Oct-4 protein level was recovered to a level close to the control. These data reveal that Wwp2 promotes both Oct-4 ubiquitination and degradation *in vivo*. In contrast, Wwp2-CA did not affect Oct-4 ubiquitination significantly, either in the presence or absence of MG132 (*lanes 6 and 7*). Moreover, the Oct-4 protein level was not reduced by overexpression of Wwp2-CA (*lane 6*, *middle panel*), further confirming the specific effect of Wwp2 E3 ligase



activity on Oct-4 ubiquitination and steady-state protein level. In contrast, there was no detectable ubiquitination when Wwp2 and His-tagged Ub were coexpressed in the cells (Fig. 5B, top panel, lane 2), suggesting that the ubiquitination detected in the assay is specifically to Oct-4. The protein level of Wwp2 in the cell lysates was also determined (bottom panel). These results indicate that Wwp2 can function as an E3 ligase of Oct-4, regulating ubiquitination and protein level of intracellular Oct-4. Besides, the cysteine at 838 residue of Wwp2 plays an essential role in this process, although its mutation had no effect on the association between Oct-4 and Wwp2 (Fig. 4A).

Ub-Oct-4 Fusion Inhibits Transcriptional Activity of Oct-4—The exact nature of the Ub modification of Oct-4 is not clear. It was previously reported that fusion of a single Ub to the amino terminus of LexA-VP16 restores its transcriptional activation in *Met³⁰*-null yeast (33). We would like to know what the functional consequence is if one copy of Ub is in-frame fused to the amino terminus of Oct-4. Thus, a molecule that mimics the monoubiquitinated form of Oct-4 was designed and then the vectors encoding either wild type Oct-4 or the *Ub-Oct-4* fusion protein (*Ub-Oct-4*) were transfected into HEK 293 cells together with the 6-PORE-luciferase reporter, which contains six copies of the octamer motif sequence (Oct-4 binding site) from the first intron of osteopontin (34). Luciferase activities and Oct-4 protein expression level in HEK 293 cells were determined. As shown in Fig. 6A, wild-type Oct-4 displayed a dose-dependent activation of the 6-PORE reporter. However, single Ub conjugation to Oct-4 dramatically abolished this activation at higher dosages. The fact that direct fusion of Ub to Oct-4 is sufficient to inhibit its transcriptional activity implies that the transcriptional activity of Oct-4 could be regulated by Ub-

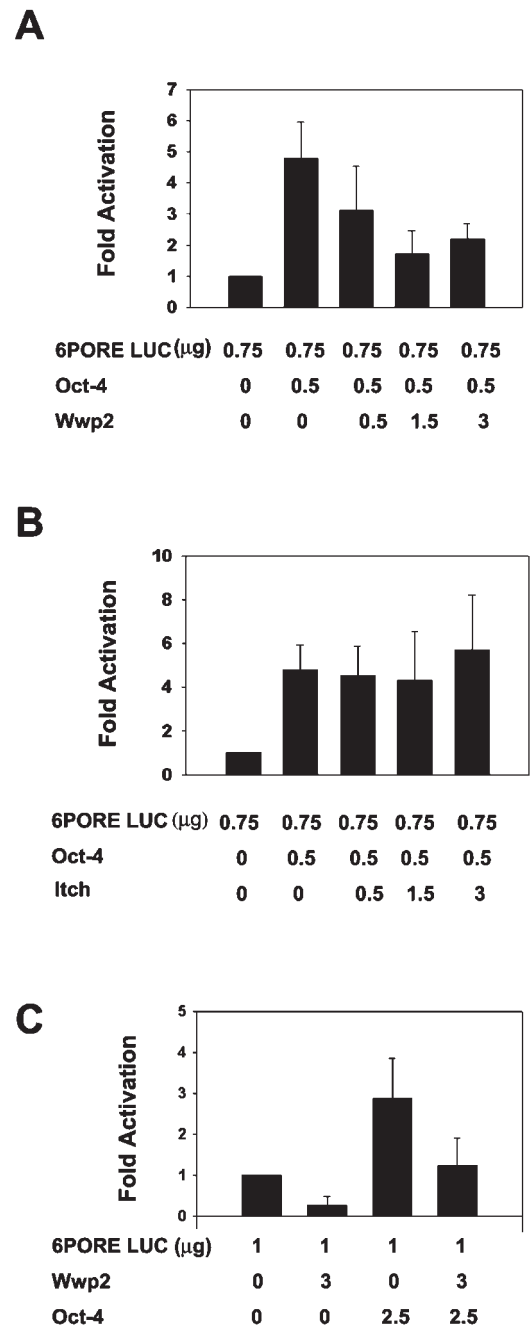


FIG. 7. **Wwp2 inhibits transcriptional activity of Oct-4.** A, 6-PORE-luciferase (*LUC*) reporter alone or in combination with Oct-4 or Wwp2 was transiently transfected into HEK 293 cells, and the luciferase activity was measured. pSV- β -Galactosidase plasmid was used as an internal standard to normalize the luciferase activity. B, the same experiments as in A were performed with Itch. C, the reporter assays were performed in CGR8 ES cells.

posttranslational modification. On the other hand, the invariant steady-state levels between Oct-4 and *Ub-Oct-4* show that it is ubiquitination of Oct-4, rather than proteolysis, that accounts for Oct-4-transcriptional inhibition under this condition (Fig. 6B).

Wwp2 Negatively Regulates Transcriptional Activity of Oct-4—To characterize the effect of Wwp2 on the Oct-4-transcriptional activity, transient cotransfection assays with Oct-4 and different amounts of Wwp2 expression vectors together with the 6-PORE-luciferase reporter vector were performed in HEK 293 cells. Fig. 7A shows that Oct-4 activated the expression of the reporter for ~5-fold. However, Wwp2 caused a dose-

pendent inhibition of Oct-4-activated expression of the reporter when Oct-4 and Wwp2 were coexpressed in these cells. As a control, a closely related E3 ligase, Itch (35), was tested in the same assay. As shown in Fig. 7B, Itch had no inhibitory effect on the Oct-4-activated 6-PORE reporter, indicating that the inhibition of Oct-4-activated 6-PORE reporter is specifically conferred by Wwp2, rather than being a common attribute of HECT domain-containing E3 ligases.

Finally, we determined whether Wwp2 could regulate the transcriptional activity of endogenous Oct-4 in ES cells. CGR8 ES cells were transiently transfected with just the 6-PORE-luciferase reporter or in combination with Oct-4 or Wwp2 or both. As shown in Fig. 7C, transfection with Wwp2 caused a decrease in the reporter activity by 70% compared with the reporter alone. As expected, Oct-4 significantly increased the reporter activity. However, cotransfection of Oct-4 with Wwp2 reduced reporter activity significantly. These results demonstrate in ES cells that Wwp2 negatively regulates transcriptional activity of both endogenous and overexpressed Oct-4.

DISCUSSION

This study identified in mouse ES cell an intriguing E3 ubiquitin-protein ligase, Wwp2, which specifically interacted with ES cell-specific transcription factor Oct-4 and promoted its ubiquitination both *in vitro* and *in vivo*. To our knowledge, this is the first demonstration that Oct-4 ubiquitination occurs. Moreover, we provided evidence that ubiquitin-Oct-4 fusion protein was transcriptionally less active than the unmodified protein. These results reveal that the functional status of Oct-4, in addition to its expression level, dictates its transcriptional activity.

ES cell lines derived from the inner cell mass are considered to be the immortal *ex vivo* counterpart of early embryo stem cells and represent a unique tool for studying scientific and medical issues. However, how pluripotency and self-renewal are sustained in these cells are far from being understood completely. Transcriptional factors Oct-4, Sox2 (36), FoxD3 (37, 38), STAT3 (39, 40), and newly identified Nanog (41, 42) work in concert to support stem cell pluripotency and self-renewal. Recently, it was demonstrated that bone morphogenetic protein induction of Id proteins via Smad pathway suppresses differentiation sustains embryonic stem cells self-renewal in collaboration with Stat3 (43). Among these factors, Oct-4 plays a specific role in blocking differentiation into trophoblast, whereas Nanog (and activated STAT3) may block differentiation into primitive endoderm and germ layers. Importantly, the functions of these master transcriptional factors are dose-dependent. Overexpression of Oct-4 induces differentiation in ES cells and possibly also in the nascent hypoblast *in vivo*, whereas a marked decrease in Oct-4 expression triggers dedifferentiation to trophectoderm (8). These effects of variations in Oct-4 expression suggest that the level of Oct-4 expression has to be tightly regulated within a narrow range to maintain pluripotent cell phenotype.

Protein-posttranslational modification by ubiquitination is a major mechanism by which cells regulate the activity of specific proteins. The ubiquitin-proteasome pathway has been found to function in a variety of cellular processes including differentiation, proliferation, apoptosis, and pathogenesis of diseases (44–46). However, the role of this pathway in ES cell self-renewal remains poorly characterized. We obtained substantive evidence that Wwp2 functions as an E3 ubiquitin-protein ligase of Oct-4. The evidence is noted as follows. 1) Wwp2 and Oct-4 form a stable complex both *in vitro* and *in vivo*. 2) Wwp2 directly catalyzes Oct-4 ubiquitination. 3) The enzymatic reaction is dependent on ubiquitin thioester bond formation at the active-site cysteine. Therefore, Oct-4 might be a physiological

target for the Wwp2 E3 ligase. Our results show that the integrity of HECT domain in Wwp2 is essential for its promotion of Ub conjugation to Oct-4 but is dispensable for its association with Oct-4. The region of Wwp2 involved in binding Oct-4 is within its four WW domains. These results are consistent with a two-domain model for HECT domain-containing E3 function in which WW domains determine substrate specificity, whereas the HECT domain catalyzes Ub conjugation to its substrate.

Although the best-studied function of ubiquitination is its role in protein degradation, more recent experiments reveal that under certain circumstances ubiquitination of transcription factors, independent of proteolysis, is required for the function of some transcription factors (47–49). It is generally considered that mono-Ub is a regulator of the location and activity of diverse cellular proteins, whereas multi-Ub chains mediate protein destruction by the proteasome (16). In this study, we found that Wwp2 promoted Oct-4 ubiquitination and degradation in the presence of exogenously overexpressed Ub and that Wwp2-induced degradation of Oct-4 was blocked by MG132, a potent inhibitor of proteasome function, suggesting that Wwp2 can promote the degradation of Oct-4 by the proteasome pathway under these experimental conditions. On the other hand, our data show that direct fusion of Ub to Oct-4 inactivated its transactivation function, implying a direct role for ubiquitination in Oct-4-transcriptional repression. However, it seems that the inactivation of Oct-4-transcriptional activity by this fusion protein was not because of increased Oct-4 degradation (Fig. 6B), suggesting a proteolysis-independent mechanism. We currently do not know what the mechanisms are for Wwp2 to inhibit transcriptional activity of Oct-4 in ES cells. The observation that direct fusion of Ub to Oct-4 is sufficient to inhibit its transcriptional activity suggests the inhibitory effect of Wwp2 on Oct-4-transcriptional activity could be mediated, at least in part, by promoting Ub conjugation to Oct-4. It is intriguing to consider that Wwp2 could catalyze monoubiquitination or polyubiquitination of Oct-4 depending on the intracellular concentration of Ub and Wwp2. Our hypothesis is supported by a recent report that p53 may face two alternative fates depending on Mdm2 levels. Low levels of Mdm2 activity induce monoubiquitination and nuclear export of p53, whereas high levels promote polyubiquitination of p53 and degradation within the cell nucleus (50, 51). Further studies are needed to determine the mechanism by which Oct-4 ubiquitination might inhibit its transcriptional activity.

Identification of Wwp2 as an E3 Ub-protein ligase of Oct-4 provides an important mechanism to maintain Oct-4-transcriptional activity in a narrow range. Especially, Wwp2 protein level was decreased in a similar pattern to Oct-4 when ES cells were induced into differentiation with retinoic acid, implying that the interaction between these two proteins might play a role in maintaining ES cells in undifferentiated status. However, as usual, the finding raises more questions than what have been answered. It is very important to determine whether Wwp2 promotes Oct-4 ubiquitination or not in ES cells and whether Wwp2 plays any role in ES cell self-renewal. Stable cell lines with overexpressed Wwp2 or Wwp2-specific RNA interference-treated cells grew poorly, which made our functional studies difficult. We are currently generating inducible Wwp2-overexpressed ES cell lines to study the function of Wwp2 in proliferation and differentiation of ES cells.

In conclusion, we have identified Wwp2 as a unique Oct-4-associated HECT domain-containing protein and have shown that Wwp2 negatively regulates Oct-4-transcriptional activity. Importantly, it was first found that transcriptional activity of Oct-4 is subjected to regulation by its ubiquitination state.

These findings have significant implications for a potential pathway to regulate Oct-4-transcriptional activity and predict that other cellular regulatory proteins can be modified and functionally regulated in a similar manner.

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Wwp2, an E3 Ubiquitin Ligase That Targets Transcription Factor Oct-4 for Ubiquitination

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