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 posttranslationally 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 is also present in cultured undifferentiated embryo cell lines including embryonic stem (ES) 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 trophodermal 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 carboxy terminus) domain, which provides the Ub ligase enzyme activity. E3 ligases of Nedd4 family...
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 direct protein ubiquitination 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 transcription-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 Oct-4 cDNA was a kind gift of N. G. Copeland. Hemaggulutinin-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 AK090295) was amplified by reverse transcription-PCR from the D3 mouse ES cell line. For expression of Wwp2 and Oct-4 in 4CR5 ES cells (a kind gift from A. Smith), the cDNA sequences were cloned into a modified X-PNT 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 μl 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 Spectrometry for Protein Identification—The protein in-gel digestion and protein identification by nano-high pressure liquid chromatography-mass spectrometry was carried out according to the procedures described previously (27).

Northern and Western Blot Analyses—A mouse multiple tissue Northern blot (Kingeac) was hybridized with digoxin (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 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.
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 evolutionally 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 μM 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
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. 3A, 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. 3B, 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. 3C). 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 (including 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. 4A, bottom panel. As shown in Fig. 4A, top panel, in addition to full-length Wwp2, WW domains bound to Oct-4 directly in vitro. 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. 4B, bottom panel). GST pull-down assays with His-Wwp2 were performed. As shown in Fig. 4B, 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 883 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. 
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 performed. As shown in Fig. 5A, 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, and Wwp2 (lane 5 in Fig. 5A, 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-
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. 5B, 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-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-de-
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 could 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 to differentiate 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 status.
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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