Phosphorylation of Williams Syndrome Transcription Factor by MAPK Induces a Switching between Two Distinct Chromatin Remodeling Complexes*

Hiroyuki Oya1, Atsushi Yokoyama1‡, Ikuo Yamakoa1, Ryoji Fujik1‡, Masayoshi Yonezawa1, Min-Young Youn1, Ichiro Takada1, Shigeaki Kato1‡, and Hirochika Kitagawa1‡

From the 1Institute of Molecular and Cellular Biosciences, University of Tokyo, Tokyo 113-0032 and 2Department of Biology and Chemistry, Institute of Applied Life Sciences, University of Tsukuba, Ibaraki 305-8571, Japan

Changes in the environment of a cell precipitate extracellular signals and sequential cascades of protein modification and elicit nuclear transcriptional responses. However, the functional links between intracellular signaling-dependent gene regulation and epigenetic regulation by chromatin-modifying proteins within the nucleus are largely unknown. Here, we describe novel epigenetic regulation by MAPK cascades that modulate formation of an ATP-dependent chromatin remodeling complex, WINAC (WICH Including Nucleosome Assembly Complex), an SWI/SNF-type remodeling complex containing Williams syndrome transcription factor (WSTF). WSTF, a specific component of two chromatin remodeling complexes (SWI/SNF-type WINAC and ISWI-containing complexes), was phosphorylated by the stimulation of MAPK cascades in vitro and in vivo. Ser-158 residue in the WAC domain, located close to the N terminus, is a major phosphorylation target. The WSTF mutant (WSTF-S158A) lacking the phosphorylation of this residue was impaired in maintaining the association between WSTF and core BAF complex components, thereby markedly altering WINAC-dependent transcription and recovery from DNA damage mediated by WINAC. WINAC mediates a phosphorylation-dependent step in epigenetic regulation, and a MAPK-dependent switching mechanism between the two functionally distinct WSTF-containing complexes might underlie the diverse functions of WSTF in various nuclear events.

Chromatin structure is intimately involved in the regulation of gene expression. The dynamics of chromatin structure are tightly regulated through multiple mechanisms such as histone modification, chromatin remodeling, histone variant incorporation, and histone eviction. Chromatin reorganization is performed by nuclear chromatin-modifying complexes to allow effector proteins (transcription factors) access to DNA (4). Several classes of chromatin-modifying complexes have been characterized (6). One class is a histone-modifying complex, and the other class is an ATP-dependent chromatin remodeling complex. The latter is responsible for regulating nucleosomal DNA structure, facilitating or preventing access to genetic information (7, 8). MAPK cascades in combination with other factors control these activities in determining the impact of a complex (6, 10, 11). Although several steps of the gene regulation can be modulated by various signaling molecules, the main regulatory mode is through modifications of transcription factors by their downstream effectors (12). Several transcription factors, including nuclear receptors, have already been reported as modification targets (13, 14). A detailed understanding of the regulatory mechanisms controlling transcription together with the reorganization of chromatin structure is lacking.

Vitamin D receptor (VDR)3 is a member of the steroid/thyroid hormone receptor superfamily regulating bone metabolism, calcium homeostasis, and cell differentiation by binding with 1α,25-dihydroxyvitamin D3 (D3), a physiologically

*This work was supported in part by a grant from the Encouraging Development of Strategic Research Centers, Special Coordination Funds for Promoting Science and Technology, Ministry of Education, Culture, Sports, Science and Technology, Japan (to H. K. and S. K.).

1 To whom correspondence may be addressed. Tel.: 81-3-5841-8478; Fax: 81-3-5841-8477; E-mail: uskato@mail.ecc.u-tokyo.ac.jp.

2 To whom correspondence may be addressed. Tel.: 81-3-5841-8478; Fax: 81-3-5841-8477; E-mail: hirokita-tky@umin.ac.jp.

3 The abbreviations used are: VDR, vitamin D receptor; MAPK, mitogen-activated protein kinase; WSTF, Williams syndrome transcription factor; WINAC, WICH including nucleosome assembly complex; ISWI, imitation switch; BAF, BRG1-associated factor; ERK, extracellular signal-regulated kinase; JNK, Jun N-terminal kinase; ChIP, chromatin immunoprecipitation; MEF, mouse embryonic fibroblast; CHAPS, 3-(3-cholamidopropyl)dimethylammonio)-1-propanesulfonic acid; GST, glutathione S-transferase; RT, reverse transcription; h, human; D3, 1α,25-dihydroxyvitamin D3; MMS, methyl methanesulfonate.
cally active form of vitamin D (15–17). Like other nuclear receptors, VDR serves as a ligand-dependent transcription factor that requires distinct classes of co-regulators and multiprotein co-regulator complexes to initiate D3-induced chromatin reorganization (18). These complex appear to modify chromatin configuration by controlling nucleosomal rearrangement and enzyme-catalyzed modifications of histone tails (19, 20). As for VDR, many types of co-regulator complexes have been identified thus far, including p160 family histone-acylating complexes, DRIP-TRAP complexes, and ATP-dependent chromatin remodeling complexes (21, 22).

WINAC is a SWI/SNF-type ATP-dependent chromatin remodeling complex that we recently identified as a VDR-interacting complex (1). WINAC, a component of WINAC, is indispensable for gene regulation by VDR through its chromatin remodeling activity (2, 3, 21, 22). The physiological role of this complex has been clarified in heart development as well as calcium metabolism through analysis of WINAC-deficient mice that lack WINAC-mediated regulation of transcription (3). WINAC constitutes another complex designated WICH (WINAC-ISWI chromatin remodeling complex) (23). WICH serves as an ISWI-type chromatin remodeling complex, which is responsible for recovery from DNA damage at various steps (3, 24).

In this study, we identified a novel type of intracellular signal-dependent epigenetic regulation mediated by WINAC, which is conceivably required for proper WINAC function by modulating WICH function. MAPK-dependent phosphorylation of WSTF is required for maintaining the complex property of VDR as an ATP-dependent chromatin remodeling complex. WINAC-mediated transcriptional property of VDR was confirmed by several ways. However, the effect of WINAC-mediated by WICH function is a novel nuclear mediator of MAPK signaling cascades for switch the two chromatin remodeling complexes.

EXPERIMENTAL PROCEDURES

Materials

Plasmids—The expression vectors for WINAC and WINAC-S158A mutants was constructed with pCAGGS (WAC-WSTF plasmid) (1) as a template vector. The primer pairs for S158A and S158E mutants was constructed with a site-directed mutagenesis kit (Stratagene, La Jolla, CA) using a pCAGGS-NcoI-WAC-WSTF plasmid (1) as a template vector. The primer pairs for S158A and S158E mutants were 5′-GGTGCGGCTGTGATGCTCAATCAA-3′ (forward) and 5′-CACCCTGACACATCAAGGAC-3′ (reverse), respectively. pGEX vectors coding GST-WSTF mutants, such as WAC_WSTF/Aclf/βbnpq46 (amino acids 1–162), WSTFm1 (amino acids 162–576), DDT (amino acids 576–669), WSTFm2 (amino acids 669–1134), WAKZ (amino acids 1134–1185), PHD (amino acids 1185–1296), and Bromo (amino acids 1296–1495) were described in our previous paper (2). The pGEX-WAC-S158A mutant was also constructed with the site-directed mutagenesis kit using pGEX-4T-WAC (amino acids 1–162) with the same primers as described above. The expression vector for activated transforming growth factor-β receptor (TβR-I(TD)) was kindly provided by Dr. Kohei Miyazono as described in our previous paper (25).

Antibodies—The antibodies used were as follows: αFLAG (F7425, Sigma); αWSTF (W1107, United States Biological Corp.); αBRG1 (H-88, Santa Cruz Biotechnology, Santa Cruz, CA); αBRM (E1, Santa Cruz Biotechnology); αBAF250 (PSG3, Santa Cruz Biotechnology); αBAF170 (H-116, Santa Cruz Biotechnology); αBAF155 (H-76, Santa Cruz Biotechnology); αBAF60a (10998-2-AP, Proteintech Group, Inc., Chicago); αINI1 (H-300, Santa Cruz Biotechnology); αhSNF2h (ab3749, Abcam, Cambridge, United Kingdom); αVDR (PP-H4537-00, Perseus Proteomics, Inc., Tokyo, Japan); α-phosphoserine (37430, Qiagen, Valencia, CA); α-Ch3 (06-549); H3K9me3 (8898-100); and H3K4me3 (8580-100) (Upstate, Millipore, Billerica, MA).

Kinase Inhibitors—ERK inhibitor (U0126), JNK inhibitor (SP600125), and Akt inhibitor (Akt inhibitor X; 124020) was purchased from Santa Cruz Biotechnology (number HTB-22). Cell Culture and Transfections—MCF7 cells or MEFs were maintained in phenol red-free Dulbecco’s modified Eagle’s medium supplemented with 10% charcoal/dextran-treated fetal bovine serum and antibiotics (Invitrogen). All culture media were supplemented with 5% CO2 (6% CO2 for differentiation). For preparation of the media, the conditioned medium (CM) was washed with 1% Nonidet P-40, followed by the addition of 0.2% Neutral Red (Life Technologies, Carlsbad, CA), 10% FBS, and 1 ml of phenylmethylsulfonyl fluoride (PMSF). Cells were cultured for 96 h at 37 °C. Luciferase was used as a reference to normalize transfection efficiencies in all experiments. All values are means ± S.D. from at least three independent experiments (1–3).

In Vitro Kinase Assay—GST fusion proteins were expressed in Escherichia coli and were bound to glutathione-Sepharose 4B beads (GE Healthcare) as described previously (1–3). One µg of recombinant ERK1 (catalog number 454849, Calbiochem), JNK2 alpha (catalog...
WSTF Is a MAPK-dependent Phosphoprotein

**FIGURE 1.** Stimulation of MAPK signaling cascades are required for the co-activation function of WSTF for D3-dependent transcriptional property of VDR. A, co-activation function of WSTF for D3-dependent VDR transcription function requires the stimulation of MAPK signaling pathways. MCF7 cells incubated in the 24-well trays were transfected with pM-VDR-DEF (15 ng), pcDNA3-WSTF, or empty pcDNA3 vector (45 ng), pGL3-17m2g (150 ng), and 10^7 MCF7 cells. The phosphorylation levels of VDR in MCF7 cells were examined by Western blot. Three 10-cm culture dishes of MCF7 cells were treated with inhibitor, U0126; WSTF deletion mutants or GST-WAC-S158A in 20^6 medium was removed and changed to fresh medium containing 1% fetal bovine serum, 10^{-7} M p38 inhibitor, 10^{-7} M Akt inhibitor, 10^{-5} M and Akt inhibitor, 10^{-5} M were added to the each well of lysis buffer (Promega), and luciferase activities were analyzed. The error bars indicate standard deviations. All measurements were done in triplicate. B, phosphorylation levels of VDR in MCF7 cells were examined by Western blot with α-VDR and α-β-actin were used as loading controls.

Maintenance of the WSTF-S158A Stably Expressing Cells and WINAC components (1). Briefly, about 10^9 cells of each stable transformant were harvested, and the nuclear extracts (80 mg) were prepared by the method initially described by Dignam et al. (34). One hundred μl of hSNF2h antibody was added to the nuclear extracts followed by batch collection with 500 μl of FLAG peptide (Sigma) for the following assays. Western blotting was performed with the phospho-protein purification kit (catalog number 37101, Qiagen) following our standard protocol. The purification of phosphoproteins was performed with the phospho-Ser/Thr column and eluted following the manufacturer’s protocol (33). Briefly, 10 10-cm culture dishes of MCF7 cells were lysed with 10 ml of lysis buffer containing 0.25% CHAPS. The cell lysates were loaded onto the anti-phospho-Ser/Thr column and eluted following the manufacturer’s protocol (Qiagen). The eluates were then subjected to Western blot.

The purification of partially purified WINAC was done following our previous paper with some modifications to remove WICH components (1). Briefly, about 10^9 cells of each stable transformant were harvested, and the nuclear extracts (80 mg) were prepared by the method initially described by Dignam et al. (34). One hundred μl of hSNF2h antibody was added to the nuclear extracts followed by batch collection with 500 μl of protein G-Sepharose (GE Healthcare). After collecting the resin on a 10-ml column, the flow-through fraction was next transferred to an anti-FLAG M2 affinity resin (Sigma) column (400-μl bed volume) and eluted from the resin with 400 μl of 300 μg/ml FLAG peptide (Sigma) for the following assays.

ATPase Assay

An ATPase assay was performed following the previous report (35). Briefly, the 5-μl reaction mixture containing 10 mm HEPES (pH 7.6), 50 mm KCl, 0.1 mm EDTA, 2 mm MgCl2, 0.5 mm dithiothreitol, 7.5% glycerol, 0.5% Nonidet P-40, 30 μm cold...
ATP, 5 μCi of [α-32P]ATP, and WINAC complexes from the stable transformant expressing FLAG-WSTF or FLAG-WSTF-S158A mutant (as described in Fig. 5, A and C) was incubated at 37 °C for 30 min. Hydrolyzed ATP and ADP were separated by TLC on polyethyleneimine-cellulose plates (Sigma). A 1-μl aliquot of the reaction mixture was spotted onto the plate, and TLC was carried out in 0.75M KH₂PO₄. Plates were allowed to dry and assessed by autoradiography.

ChIP Assay

ChIP assay was performed as described previously (1, 2, 36). Briefly, the cross-linked and sheared chromatin prepared from 10⁶ cells was subjected to immunoprecipitation with 2 μg of each antibody against the indicated proteins. The following procedure was performed using the ChIP assay kit (Upstate) according to the manufacturer’s instructions. Immunoprecipitated chromatin was subjected to PCR using the primer pairs described below. The primer pairs for the promoters of 25(OH)₂-hydroxylase and 25(OH)₁-hydroxylase were 5′-GGGGAGGCGCGTTCGAA-3′ (forward) and 5′-TCCTATGCCAGGGAC-3′ (reverse) and 5′-ATTCCCATGTCCTGGAAGAG-3′ (forward) and 5′-CAGTGAGC-CCAGGCCCTTA-3′ (reverse), respectively (1).

Quantitative RT-PCR

Total RNA was extracted with TRIzol (Invitrogen), and cDNA was synthesized using SuperScriptIII reverse transcriptase (Invitrogen). Reverse transcription of 2 μg of total RNA was carried out with 0.2 μg of oligo(dT) primer for 50 min at 50 °C. Quantitative RT-PCR was performed using SYBR Premix EX Taq (Takara) according to the manufacturer’s instructions. Predesigned quantitative RT-PCR primer sets were purchased from Takara. Experimental samples were matched to a standard curve generated by amplifying serially diluted product using the same PCR protocol. To correct for variability in RNA recovery and efficiency of reverse transcription, glyceraldehyde-3-phosphate dehydrogenase cDNA was amplified and quantified in each cDNA preparation (3, 31, 37).

Cell Survival Assay

All experimental procedures were conducted as described in our previous reports (3, 38). MEF cells from WSTF−/− and wild type mice were incubated in 60-mm dishes at 40% confluency (4 × 10⁵ cells/dish). The indicated expression vectors were transfected with Lipofectamine Plus reagent (Invitrogen). After 24 h, transfected cells were treated with medium containing 0.02% methyl methanesulfonate (MMS) for 1 h, washed with

FIGURE 2. WSTF is phosphorylated by MAPKs in vitro. A, schematic representation of the deletion mutants of WSTF fused to GST. Mutations of Ser-158 converted to alanine (S158A) and glutamate (S158E) are indicated by the square, which were described in the following experiments. B, WAC domain of WSTF is phosphorylated by ERK1 in vitro. The upper image shows the CBB staining. Recombinant active-ERK1, bacterially expressed GST-WSTF deletion mutants were purified with glutathione-Sepharose beads and used for the in vitro kination assay as a substrate of ERK1 protein. The arrow shows the position of WAC domain of WSTF phosphorylated by ERK1. The lower image shows the Autoradiography of the in vitro kination assay. GST-WSTF deletion mutants bound to glutathione-Sepharose beads were incubated with recombinant active-ERK1 in the presence of [γ-32P]-labeled ATP, and the phosphorylation of WSTF mutants was detected by autoradiography. The asterisk indicates the background signals. C, serine 158 of WSTF is the target amino acid residue of MAPK-dependent phosphorylation. In vitro kination assay was performed using recombinant active-ERK1, JNK2, p38, and Akt1 proteins. Upper image shows the amount of GST, GST-WSTF, and GST-WSTF-S158A used as phosphorylation substrates. The lower images show the 32P-labeled GST-WSTF mutants phosphorylated by the indicated protein kinases. Recombinant active Akt1 was applied as a negative control for the kination reaction by MAPK effector kinases.
phosphate-buffered saline, and maintained for 4 days in fresh medium. Surviving cells were then counted. Percent survival rate is the rate of the total cell number in the dish under indicated conditions versus the cell number in the dish with non-MMS treatment. All values are means ± S.D. from six independent experiments.

RESULTS

WSTF Is a MAPK-dependent Phosphoprotein in Vitro and in Vivo—Our recent analysis of WSTF-deficient animals (3) showed that WSTF contributes to various biological events presumably through the chromatin remodeling activity of the two complexes, WINAC and WICH (1, 23). However, the links between the physiological impact of WSTF and the ATP-dependent chromatin remodeling activities of these complexes are still largely unknown. We hypothesized that an unrecognized intracellular signaling pathway might mediate both the physiological functions of WSTF and the chromatin remodeling activities.

To identify intracellular signals affecting the function of these complexes in vivo, we tested inhibitors against several cellular signaling pathways using our established reporter assay system, Gal4-fused VDR (Gal-VDR) and WSTF in MCF7 (1–3). As shown in Fig. 1A, induction of the activated transforming growth factor-β receptor (TβR-I(TD)) potentiated the 1,25(OH)2D3(D3)-dependent VDR transcriptional property regardless of the presence or absence of WSTF as reported previously (compare lanes 21–24 with lanes 1–4) (25, 39). Among the tested inhibitors against the signaling pathways, an Akt inhibitor (Akt inhibitor X) did not affect the co-activation function of WSTF (Fig. 1A, compare lanes 17–20 with lanes 1–4).

Nevertheless, three inhibitors for MAPK signalings, which interfere with distinct downstream pathways, strongly decreased the co-activation function of WSTF on Gal-VDR (Fig. 1A, compare lanes 5–8, 9–12, and 13–16 with lanes 1–4, respectively). MAPK cascades are known to respond to changes in the cellular environment (40, 41), and the primary regulatory effectors work through the phosphorylation of transcription factors by downstream kinases such as ERK, JNK, or p38 (12). Although MAPK cascades are known to regulate the expression level of VDR (42), the protein expression levels, as well as the phosphorylation levels (43), were not

Serine 158 is the major target of MAPK-dependent WSTF phosphorylation in vivo. Three 10-cm dish cultures of MCF7 cells were treated with indicated MAPKs inhibitors (ERK inhibitor, 10−7 M; JNK inhibitor, 10−7 M; p38 inhibitor, 10−6 M; Akt inhibitor, 10−6 M) with a 20μl-bead volume of FLAG M2-agarose. 1 h after immunoprecipitation with α-FLAG antibody and α-phospho-Ser-158 antibody, cells were treated 10−7 M D3. 16 h after treatment, western blot using α-FLAG antibody and α-phospho-Ser-158 antibody were performed. WSTF at Ser-158. MCF7 cells incubated with D3 (10−7 M) were added to the wells. 16 h after the treatments, cells were lysed with lysis buffer (Promega), and luciferase activities were analyzed. The error bars indicate standard deviations. All measurements were done in triplicate.
**WSTF Is a MAPK-dependent Phosphoprotein**

1. **WSTF Phosphorylation by MAPK Signaling Downstream Kinases Is Required for the ATPase Activity of WINAC**—To determine whether WSTF was the only target of MAPK-dependent phosphorylation in the WINAC-containing complexes, we asked whether the phosphorylation levels of components other than WSTF were affected by a MAPK inhibitor (ERK inhibitor). In this protocol, whole cell extracts of the MCF7 cells stably expressing WSTF with or without ERK inhibitor (U0126) were subjected to Western blot (Fig. 4B). Among the tested human SWI/SNF-type complexes, including WINAC and hSNF2h, the phosphorylation level of WSTF-S158A mutant cells (Fig. 3A, lanes 5–8). Indeed, the phosphorylation level of WSTF-wild type, as well as its expression level, was not altered by D3 stimulation (Fig. 3B), and the co-activation function of a phosphorylation mimic mutant of WSTF (S158E) (44) was comparable with WSTF-wild type (Fig. 3C, compare lane 2 with lanes 6–9). But the co-activation function of this S158E mutant, as well as S158A mutant, for the VDR transcriptional property was not affected by the indicated MAPK inhibitors in MCF7 cells (Fig. 3C). Taken together, we conclude that WSTF is indeed phosphorylated by the activation of the three MAPK pathways in vivo, and Ser-158 residue is the main target residue of this phosphorylation.

2. **Phosphorylation of WSTF Is Required for WINAC Function but Not for WICH Function in Vivo**—Finally, we analyzed the contribution of WSTF phosphorylation to the physiological impact of WSTF. In our previous reports, we showed that the chromatin remodeling activity of WINAC contributed to both ligand-dependent repression as well as activation by VDR (1, 2, 36, 45). To test whether MAPK-dependent phosphorylation affected these two transcriptional activities, quantitative PCR analysis was performed comparing the two stable lines. We chose two representative VDR target genes 25(OH)24-hydroxylase as a positively regulated gene and 25(OH)1α-hydroxylase as a negatively regulated gene (1, 36). Comparing the expression profiles of the genes after D3 stimulation, both the D3-depen-
WSTF is a MAPK-dependent Phosphoprotein

FIGURE 5. MAPK-dependent phosphorylation of WSTF is required for the assembly of WINAC complex. A, schematic diagram of the partial purification of the WINAC complex. 80 mg of nuclear extracts prepared from MCF7 cells stably expressing FLAG-WSTF or FLAG-WSTF-S158A mutant were subjected to immunoprecipitation with α-hSNF2 antibody to remove WICH complex. The immunocomplexes fused to α-FLAG antibody were incubated with [γ-32P]ATP and unhydrolyzed [γ-32P]ADP are indicated on the left. The α-FLAG antibody column and flow-through of the α-hSNF2 antibody column were subjected to Western blot using hSNF2 antibody. α-hSNF2 antibody could be detected in the immunocomplex of the α-FLAG antibody column, whereas it could not be detected in the flow-through fraction of the α-hSNF2 antibody column (lanes 1–2 vs. lane 5, respectively). B, confirmation of WICH complex depletion. WSTF-wild type and WSTF-S158A mutant MEF cells were transiently transfected with WSTF-wild type and S158A mutant. Western blots of αSNF2h antibody column and flow-through of the αSNF2h antibody column were subjected to Western blot using αSNF2h antibody column. αSNF2h antibody could be detected in the immunocomplex of the αSNF2h antibody column, whereas it could not be detected in the flow-through fraction of the αSNF2h antibody column (lanes 1–2 vs. lane 5, respectively). C, WINAC components. Western blots of αSNF2h antibody column and flow-through of the αSNF2h antibody column were subjected to Western blot using indicated antibodies. Indicated BAF components (αBAF155, αBAF170, αBAF250, αBAF60a, and αINI1) could be detected in the immunocomplex of the αSNF2h antibody column, whereas they could not be detected in the flow-through fraction of the αSNF2h antibody column. D, schematic diagram of the partial purification of the WINAC complex. 80 mg of nuclear extracts prepared from MCF7 cells stably expressing cells nuclear extracts were subjected to Western blot using indicated antibodies. Anti-hSNF2h-Ab, anti-αSNF2h antibody column; α-FLAG column, α-FLAG antibody column; FT, flow-through of the α-FLAG antibody column; NE, nuclear extracts; Elute, eluate of the α-FLAG antibody column; αSNF2h column, αSNF2h antibody column.

DISCUSSION

Nuclear events such as transcription, DNA replication, and DNA repair are now believed to be orchestrated by strict epigenetic controls through reorganization of chromatin structure. Some of the protein complexes regulating changes in chromatin structure have been shown to link with intracellular signaling cascades (26, 31, 32, 46, 47). However, the underlying mechanisms of signal-dependent epigenetic changes are not fully understood. For example, in the case of chromatin remodelers, although some components are known to be recruited by specific transcription factors in a signal-dependent manner (48, 49), the manner in which the specific combinations of components assemble on DNA has remained elusive (21, 22). In this study, signal-dependent complex stabilization was observed by the phosphorylation of a specific component, WSTF. This result implies novel signal-dependent regulation of complex assembly by a protein modification downstream of MAPK signaling cascades (50).

Chromatin remodeling complexes work at various situations to facilitate access of the biological effectors to the target regions of the genome through altering the adjacent chromatin

for the full activity of VDR as a ligand-dependent transcription factor.

Next we determined whether the phosphorylation of WSTF contributed to WICH function. From our previous analysis, the function of WICH appears obvious in the recovery from DNA damage in MEF cells from WSTF−/− animals (3). Thus, we tested the cell survival rate after DNA damage with overexpression of WSTF-wild type and WSTF-S158A mutant in the MEFs from WSTF−/− mice as performed previously (3). As expected, clear contribution of WICH to the recovery from DNA damage was seen (Fig. 7A, compare lanes 2 and 3 and 4 and lane 5 with lane 6, respectively). This result implies novel signal-dependent regulation of complex assembly by a protein modification downstream of MAPK signaling cascades (50).
status (8, 51). Considering the known roles of the specific components of each chromatin remodeling complex (52–55), we believe that WSTF, as a specific component of WINAC, specifically works as a sensor of the various intracellular signalings for turning on the chromatin remodeling activity when required. Indeed, in our first screening, several inhibitors against various intracellular signaling cascades affected the co-activation function of WSTF (data not shown). Further mechanical analysis seems essential to understand the biological impacts of WSTF as an epigenetic determinant under various extracellular stresses when distinct intracellular signaling cascades are activated.

The physiological impact of MAPK-dependent modification of WSTF can be appreciated when WSTF-deficient mice are considered (3). WSTF is a shared component of two chromatin remodeling complexes, WINAC and WICH (1, 23). WSTF-deficient mice have cardiovascular abnormalities that are presumably due to WINAC-dependent malfunction of cardiac transcription factors. On the other hand, DNA damage repair was also impaired probably due to the dysfunction of WICH (3). From our present analysis, MAPK-dependent phosphorylation of WSTF was found indispensable for proper WINAC function but not for WICH function. Thus, we surmise that MAPK-dependent regulation of WINAC function must have contributed to the abnormal cardiac development characteristic of WSTF-deficient mice.

It is also well known that the activation of distinct MAPK pathways has a different biological impact in heart tissues (56, 57). ERK pathway is essential for the heart development and is consequently related to certain hereditary diseases (58, 59), whereas p38 and JNK pathways, as well as ERK pathway, are for adaptational myocyte growth after birth (60). Thus, we speculate that the phosphorylation of WSTF by each MAPK downstream effector kinase has a distinct biological impact at various phases in the heart tissues. Combined with our histological analysis of the heart tissues from the WSTF-deficient mice embryos (3), it is conceivable that their phenotypes are attributed to the lack of biological impacts of MAPK signalings (presumably of ERK pathway) during the heart development, at least in part. As the WSTF-deficient mice die soon after birth (3), it seems impossible to test the role of WSTF-mediated chromatin remodeling activity at the adaptational myocyte growth in the situations such as the formation of myocardial hypertrophy or cardiac ischemia (56). For the better understanding of the contribution of each MAPK pathway to the

FIGURE 6. Phosphorylation of WSTF by MAPK downstream kinases is required for the transcriptional regulation of VDR by WINAC.

A, D3-dependent transcriptional regulation mediated by VDR was impaired in MCF7 cells stably expressing WSTF-S158A mutant. mRNA levels of VDR-targeted genes were evaluated by quantitative RT-PCR. MCF7 cells stably expressing WSTF-wild type or WSTF-S158A mutant were treated with 10−7 M D3 for the indicated time. The figures show the relative expression level of the indicated genes determined by the expression levels in the cells treated with vehicle control. The expression levels of the genes were normalized to those of glyceraldehyde-3-phosphate dehydrogenase, and error bars indicate standard deviations. All measurements were done in triplicate.

B, effect of WSTF-S158A mutation on histone tail modification as compared with WSTF-wild type. MCF7 cells stably expressing WSTF-wild type or WSTF-S158A mutant were treated with 10−7 M D3. 24 h after treatment, ChIP analysis was performed with rabbit IgG (IgG), α-FLAG antibody (FLAG), α-acetyl histone H3 (AcH3), and α-trimethylated histone H3 lysine 9 (H3K9me3) or α-trimethylated histone H3 lysine 4 (H3K4me3) antibodies. Immunoprecipitated chromatin was then subjected to PCR using indicated primer pairs for 25(OH)24-hydroxylase (24(OH)ase) or 25(OH)1α-hydroxylase (1α(OH)ase), respectively.
WSTF Is a MAPK-dependent Phosphoprotein

WSTF function at various conditions, target gene analysis might be helpful when mice selectively ablated of WSTF in hearts are available.

Considering the selective regulation of the function of two WSTF-containing complexes, MAPK-dependent phosphorylation might represent a regulatory switch for the two complexes to work properly under specific conditions (see Fig. 7D). Moreover, it is possible that combined protein modifications by other kinases or certain signaling effectors with this MAPK-dependent phosphorylation might fine-tune the two complexes to work separately at a distinct situation. A recent report suggests that WSTF acts as a tyrosine kinase in the WICH complex during DNA repair process (24). Together with our findings, intracellular signaling-induced protein modification of WSTF can modulate the enzymatic activity of WSTF itself. It is also conceivable that the modification state of WSTF defines the spec-
cific function of each WSTF-containing complex. In this regard, further study is necessary for a better understanding of the intracellular signaling-dependent function of the WSTF-containing complexes under various extracellular stresses.

Transcriptional regulation by ATP-dependent chromatin remodeling complexes has recently been examined in detail (22). For instance, highly specific phosphorylation-dependent regulation of specific SWI/SNF complex components (BRG1/BRM or BAF60a) has been reported (49, 61). Considering the protein modification-dependent regulation of these chromatin remodeling complexes, we speculate that the regulation of WSTF phosphorylation might be a clue to understanding the developmentally regulated functions of WINAC (1, 3, 6). For example, in the regulation of VDR-mediated transcription, we have already reported impaired in the D3-dependent repressive promoter as well as in the activational promoter (1, 2, 36). We have already reported that D3-dependent co-pressor-regulator with the 25(OH)1α-hydroxylase promoter is related to protein kinase C signaling as well as protein kinase A signaling (2, 36, 62). Considering the involvement of MAPK signaling pathways in this process, further co-dependent analysis of the intracellular signaling-dependent factor recruitment might lead to a comprehensive understanding of the mechanism of D3-dependent transactivation.

Acknowledgments—We thank all the members of the Kato laboratory for helpful discussions and Hiroko Yamazaki for manuscript preparation.
WSTF Is a MAPK-dependent Phosphoprotein

45. Murayama, A., Takeyama, K., Kitanaka, S., Kodera, Y., Hosoya, T., and Kato, S. (1998) Biochem. Biophys. Res. Commun. 249, 11–16
46. Yang, S. H., and Sharrocks, A. D. (2004) Mol. Cell 13, 611–617
47. Nott, A., Watson, P. M., Robinson, J. D., Crepaldi, L., and Riccio, A. (2008) Nature 455, 411–415
48. Takeuchi, J. K., Lickert, H., Bisgrove, B. W., Sun, X., Yamamoto, M., Chawengsaksophak, K., Hamada, H., Yost, H. J., Rossant, J., and Bruneau, B. G. (2007) Proc. Natl. Acad. Sci. U.S.A. 104, 846–851
49. Simone, C., Forcales, S. V., Hill, D. A., Imbalzano, A. N., Latella, L., and Puri, P. L. (2004) Nat. Genet. 36, 738–743
50. Yang, S. H., Sharrocks, A. D., and Whitmarsh, A. J. (2003) Gene 320, 3–21
51. Sif, S. (2004) J. Cell Biochem. 91, 1087–1098
52. Takeuchi, J. K., and Bruneau, B. G. (2009) Nature 459, 708–711
53. Rottbauer, W., Saurin, A. J., Lickert, H., Shen, X., Burns, C. G., Wo, Z. G., Kemler, R., Kingston, R., Wu, C., and Fishman, M. (2002) Cell 111, 661–672
54. Wang, Z., Zhai, W., Richardson, J. A., Olson, E. N., Meneses, J. I., Firpo, M. T., Kang, C., Skarnes, W. C., and Tjian, R. (2004) Genes Dev. 18, 3106–3116
55. Yoo, A. S., Staahl, B. T., Chen, L., and Crabtree, G. R. (2009) Nature 460, 642–646
56. Olson, E. N., and Schneider, M. D. (2003) Genes Dev. 17, 1937–1956
57. Ravingerová, T., Barancík, M., and Strnisková, M. (2003) Mol. Cell. Biochem. 247, 127–138
58. Tidyman, W. E., and Rauen, K. A. (2008) Expert Rev. Mol. Med. 10, e37
59. Nakamura, T., Colbert, M., Krenz, M., Molkentin, J. D., Hahn, H. S., Dorn, G. W., 2nd, and Robbins, J. (2007) J. Clin. Invest. 117, 2123–2132
60. Bogoyevitch, M. A., and Sugden, P. H. (1996) Int. J. Biochem. Cell Biol. 28, 1–12
61. Sif, S., Stukenberg, P. T., Kirschner, M. W., and Kingston, R. E. (1998) Genes Dev. 12, 2842–2851
62. Kim, M. S., Fujiki, R., Kitagawa, H., and Kato, S. (2007) Mol. Cell. Endocrinol. 265, 168–173
63. Rosenfeld, M. G., Lunyak, V. V., and Glass, C. K. (2006) Genes Dev. 20, 1465–1428