β-Transducin Repeat-containing Protein 1 (β-TrCP1)-mediated Silencing Mediator of Retinoic Acid and Thyroid Hormone Receptor (SMRT) Protein Degradation Promotes Tumor Necrosis Factor α (TNFα)-induced Inflammatory Gene Expression*

Received for publication, March 28, 2013, and in revised form, July 15, 2013. Published, JBC Papers in Press, July 16, 2013, DOI 10.1074/jbc.M113.473124

Kuo-Sheng Hsu‡, and Hung-Ying Kao‡§

From the ‡Department of Biochemistry, Case Western Reserve University, Cleveland, Ohio 44106 and the §Comprehensive Cancer Center of Case Western Reserve University and University Hospitals of Cleveland, Cleveland, Ohio 44106

Background: SMRT is a transcriptional corepressor, and β-TrCP1 is a subunit of ubiquitin E3 ligase.

Results: TNFα stimulation up-regulates β-TrCP1, which promotes SMRT polyubiquitination and proteolysis. β-TrCP1 knockdown in endothelial cells enhances SMRT occupancy on target gene promoters and decreases their expression.

Conclusion: The TNFα downstream β-TrCP1-SMRT axis derepresses SMRT-targeted genes.

Significance: Understanding the TNFα-β-TrCP1-SMRT axis in the proinflammatory response will further our understanding of inflammation-associated diseases.

Cytokine modulation of the endothelium is considered an important contributor to the inflammation response. TNFα is an early response gene during the initiation of inflammation. However, the detailed mechanism by which TNFα induces proinflammatory gene expression is not completely understood. In this report, we demonstrate that silencing mediator of retinoic acid and thyroid hormone receptor (SMRT) represses the expression of a subset of TNFα target genes in human umbilical vein endothelial cells. Upon TNFα stimulation, we observed an increase in the E3 ubiquitin ligase β-TrCP1 and a decrease in SMRT protein levels. We show that β-TrCP1 interacts with SMRT in a phosphorylation-independent manner and cooperates with the E2 ubiquitin-conjugating enzyme E2D2 to promote ubiquitination-dependent SMRT degradation. Knockdown of β-TrCP1 increases SMRT protein accumulation, increases SMRT association with its targeted promoters, and decreases SMRT target gene expression. Taken together, our results support a model in which TNFα-induced β-TrCP1 accumulation promotes SMRT degradation and the subsequent induction of proinflammatory gene expression.

Pathogen-mediated acute inflammation is recognized by the innate immune system, which is critical for repair of damaged tissue and defense against infection. By contrast, chronic inflammation in the endothelium is considered a major cause of atherosclerosis and cardiovascular diseases (1). Normally, the expression of inflammatory genes in endothelial cells (ECs) is repressed but becomes activated in response to extracellular stimuli, including infection, injury, and cytokine exposure. In the endothelium, cytokine stimulation results in induction of the expression of a subset of proinflammatory genes. Several transcription factors and transcriptional coregulators have been shown to play a key role in cytokine signaling, ensuring an appropriate inflammatory response (2, 3).

TNFα is an immediate-response cytokine secreted by the endothelium and immune cells when they sense extracellular risk signals, such as injury or infection (4, 5). The release of TNFα from ECs and immune cells in circulation not only induces the expression of adhesion molecules to recruit leukocytes to the injured lesion but also amplifies the inflammatory response via a cytokine-chemokine cascade (1, 6). These cytokines and chemokines further attract leukocytes and promote their differentiation into macrophages to maintain long-term inflammation until clearance of the risk factors is achieved (6).

To maintain homeostasis, most inflammation-associated genes are repressed under an unstimulated condition. The nuclear receptor corepressor (NCoR) and silencing mediator of retinoic acid and thyroid hormone receptor (SMRT) are two potent transcriptional corepressors that repress inflammatory response genes (7, 8). Guided by inflammation-responsive transcription factors, such as AP-1, NF-κB, and BCL-6, NCoR and SMRT establish a complex repression network to silence a set of inflammatory genes in mouse macrophages (7, 9). Disruption of the BCL-6 and NCoR-SMRT interaction in mouse or human macrophages abolishes NCoR-SMRT-mediated repression and promotes inflammatory events such as atherosclerosis (8, 10).

*This work was supported, in whole or in part, by National Institutes of Health Grants R01 DK078965 and HL093269 (to H. Y. K.).

1 To whom correspondence should be addressed: Department of Biochemistry, Case Western Reserve University and The Comprehensive Cancer Center of Case Western Reserve University and University Hospital of Cleveland, 10900 Euclid Ave., Cleveland, OH 44106-4935. Tel.: 216-368-1150; Fax: 216-368-3419; E-mail: hxk43@cwru.edu.

2 The abbreviations used are: EC, endothelial cell; NCoR, nuclear receptor corepressor; SMRT, silencing mediator of retinoic acid and thyroid hormone receptor; HUVEC, human umbilical vein endothelial cell; CIP, calf intestinal phosphatase; Ub, ubiquitin; SCF, Skp, Cullin, F-box-containing complex.
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These results suggest that SMRT is critical for the regulation of the inflammatory response in the immune system. However, SMRT function and its regulation in the endothelium in response to cytokine activation have not been thoroughly investigated.

Upon TNFα stimulation, an early response is the selective induction of inflammatory genes driven by NF-κB (11–15). Although several mechanisms underlying the activation of NF-κB have been proposed (15), a complete picture includes less well studied nuclear events. One strategy to facilitate NF-κB-mediated transactivation is the removal of repressive complexes from NF-κB target gene promoters. This is achieved by phosphorylation and nuclear export of SMRT, a NF-κB-associated transcription corepressor (16, 17). Alternatively, substantial decreases in SMRT levels through transcriptional regulation have been shown to contribute to NF-κB transactivation in human adipocytes (18). In addition to transcriptional regulation, ubiquitination-mediated proteolysis of SMRT has been suggested as a posttranslational pathway to lower SMRT expression in human umbilical endothelial cells (HUVECs).

**EXPERIMENTAL PROCEDURES**

**Plasmids and DNA Constructs**—The expression plasmids CMX-SMRT, CMX-β-TrCP1, and dominant-negative UBE2D2 (dnUBE2D2) were generated by PCR and subcloned into a cytoplasmic-megalovirus-based promoter (CMX)-HA and FLAG or Myc vectors. SMRT point mutations (3X) and deletion and truncation expression plasmids were subcloned into CMX-HA vectors or used as described previously (20). FLAG- or Myc-β-TrCP1/2 and HA-Ub were generated by PCR from a HeLa cDNA library. Site-directed mutagenesis was used to generate point mutations and deletions. The GST-β-TrCP1 and truncated GST-SMRT fusions were generated by PCR and subcloning. All clones were sequenced to confirm their identities.

**Cell Lines, Reagents, and Antibodies**—HeLa and HEK-293T cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% FBS, 50 units of penicillin G/ml, and 50 μg of streptomycin sulfate at 37 °C in 5% CO₂. HUVECs were purchased from Lonza and cultured in endothelial cell basal medium (EBM-2, Lonza) with EGM-2 SingleQuot growth supplements (Lonza). Cells of < 6 passages were used in this study. TNFα was purchased from Promega (G5421). siRNAs were purchased from Thermo Scientific, and the siRNA sequences will be provided upon request. The antibodies used were α-BCL-6 (catalog no. sc-858), α-p65 (catalog no. sc-2212), α-HA (catalog no. sc-805), and α-mouse-IgG conjugated with HRP (catalog no. sc-2005) from Santa Cruz Biotechnology; α-FLAG (catalog no. F3165), α-β-TrCP1 (catalog no. 37-3400), normal goat IgG (catalog no. 10200), Alexa Fluor 488 goat anti-rabbit (catalog no. A-11008), and Alexa Fluor 594 goat antimouse (catalog no. A-11005) from Invitrogen; α-rabbit-IgG conjugated with HRP (catalog no. 12-348) from Millipore; α-HA conjugated with HRP (catalog no. 12013819001) from Roche; and α-β-TrCP1 (catalog no. 4394) from Cell Signaling Technology. Anti-SMRT antibodies and anti-ubiquitin antibodies were purified as described previously (20, 21). The transfection reagent DharmaFECT1 (catalog no. T-2001) was purchased from Thermo Scientific.

**Transient Transfection**—Transient transfection of a total of 10 μg of expression plasmids was performed using Lipofectamine 2000 according to the protocol of the manufacturer (Invitrogen), and cells were harvested 48 h after transfection. For siRNA knockdown, a non-targeting siRNA or two independent siRNAs against β-TrCP1 or SMRT (Thermo Scientific) were transfected into HeLa cells or HUVECs using DF1 transfection reagent (Thermo Scientific) according to the protocol of the manufacturer. Cells were harvested 72 h after transfection, and total RNAs and cell extracts were prepared.

**Total RNA Extraction, RT-PCR, and Real-time PCR**—Twenty-four hours after transfection with siRNAs, HeLa cells and HUVECs were harvested, and total RNA was prepared using PrepEase RNA spin kits (USB/Affymetrix) and quantified by A₂₆₀/A₂₈₀ spectrometry. The cDNA pool was generated from each RNA sample with Superscript 3 reverse transcriptase (Invitrogen) according to the instructions of the manufacturer. The cDNAs of interest and internal controls were quantified by real-time PCR using an iCycler (Bio-Rad) platform with 2 × iQ SYBR Green Supermix (Bio-Rad) and appropriate primer sets. The PCR program was set for 40 cycles with three steps of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s. Melting curves were acquired after PCR to ensure the homogeneity of the PCR products. The relative quantities of the genes of interest were normalized to an internal control (18 S rRNA) and depicted as mean ± S.E. from three independent experiments. The primer sequences will be provided upon request.

**In Vitro Protein-Protein Interaction Assays**—GST and GST-SMRT fusion proteins were expressed in the Escherichia coli DH5α strain, and GST-β-TrCP1 was expressed in E. coli BL21 plys (Promega), purified, and immobilized on glutathione-Sepharose 4B beads. GST pull-down assays were carried out according to our published protocol (20). Briefly, immobilized GST-SMRT and GST-β-TrCP1 beads were incubated with whole cell extracts expressing FLAG-β-TrCP1 or HA-SMRT, respectively for 1 h at 4 °C. After extensive washes, pull-down fractions were subjected to SDS-PAGE followed by Western blotting with anti-HA or anti-FLAG antibodies. For phosphorylation-dependent binding assays, HA-SMRT-expressing lysates were treated with calf intestinal phosphatase (CIP) (10 units/ml, New England Biolabs) prior to incubation with immobilized GST fusion proteins for 45 min at 30 °C.

**Coimmunoprecipitation**—HEK-293T and HUVEC whole cell lysates were resuspended in NTEN buffer (20 mM Tris-Cl (pH 8.0), 100 mM NaCl, 0.1% Nonidet P-40, 1 mM EDTA, 10% glycerol, and 1 mM dithiothreitol) containing a mixture of protease inhibitors (Roche) followed by sonication. Coimmunoprecipitation was carried out using purified an anti-SMRT anti-
body (20), and immunoprecipitates were subsequently pulled down by protein A beads. The immune pellets were subjected to SDS-PAGE, followed by immunoblotting with anti-SMRT and anti-β-TrCP1 antibodies (Sigma). For communoprecipitation assays with overexpressed SMRT and β-TrCP1, HeLa cells were cotransfected with plasmids expressing FLAG-β-TrCP1, HA-SMRT, or their combination with Lipofectamine 2000. Forty-eight hours after transfection, whole cell lysates were prepared with radioimmuno precipitation assay buffer (1-PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS) with protease inhibitors and immunoprecipitated with anti-FLAG (M2) or anti-HA affinity beads (Sigma) for 4 h at 4 °C. The immunoprecipitates were subjected to SDS-PAGE, followed by immunoblotting with anti-FLAG and HRP-conjugated anti-HA antibodies (Sigma).

Immunofluorescent Microscopy—HeLa cells were cultured in 12-well plates and transfected with the indicated plasmids. Twenty-four hours after transfection, immunostaining was performed as described previously (22) using anti-SMRT or anti-FLAG antibodies and secondary antibodies conjugated with Alexa Fluor 488 or 594 (Invitrogen). Cells were mounted with DAPI (Vectashield, Vector Laboratories), and images were visualized on a fluorescent microscope (Leica). Images were captured and obtained with a camera.

In Vivo Ubiquitination Assay—HeLa cells were cultured in 10-cm dishes and transfected with 10 μg of total expression plasmids as indicated. Twenty-four hours after transfection, cells were treated with or without 50 μM MG132 (Sigma) 5 h prior to harvest, followed by cell extract preparation with NTEN buffer. Whole cell lysates were immunoprecipitated with the appropriate antibodies. After extensive washing, the affinity beads were mixed with SDS buffer and subjected to SDS-PAGE separation. To detect ubiquitination, immunopellets were subjected to Western blotting with antibodies against anti-HA (Ub) antibodies.

ChIP—ChIP assays were modified from our published protocol (23). Briefly, HUVECs were transfected with control or β-TrCP1 siRNAs for 72 h and treated with 1% formaldehyde for 10 min at room temperature. The cross-linking reaction was stopped by incubating cells with 125 mM glycine for 5 min. Cells were collected by centrifugation at 4 °C and lysed in 500 μl of nuclei lysis buffer (50 mM Tris-Cl (pH 8.1), 10 mM EDTA, 1% SDS) on ice for 10 min. Chromatin was prepared by sonicating DNA fragments to 300~700 bp, followed by 14,000-rpm centrifugation for 15 min at 4 °C. The collected supernatant was precleared with sheared salmon DNA and protein A beads at 4 °C for 2 h. After centrifugation, precleared chromatin was aliquoted and incubated with the appropriate antibodies overnight at 4 °C. The immune complex was subsequently pulled down by protein A beads and washed twice with dialysis buffer (50 mM Tris-Cl (pH 8.0), 2 mM EDTA, 2% Sarkosyl) and four times with subsequent immunoprecipitation wash buffer (100 mM Tris-Cl (pH 9.0), 500 mM LiCl, 1% Nonidet P-40, 1% deoxycholic acid). To elute the DNA-protein complex, a freshly made elution buffer (50 mM NaHCO3, 1% SDS) was mixed with the beads for 20 min at room temperature, and the eluate was collected twice by centrifuge. The eluted chromatin was incubated with 300 mM NaCl at 68 °C to reverse the DNA-protein cross-links. Meanwhile RNA was digested by 1 mg/ml RNase at 68 °C overnight, and protein was subsequently digested with 100 mg/ml proteinase K at 45 °C for 3 h. The DNA was extracted by phenol-chloroform-isoamyl alcohol mix (25:24:1). 10 μg of glycogen (Roche) was added to each sample, and the DNA was precipitated with 2.5× volume of pure ethanol in −20 °C overnight. The final DNA was pelleted by centrifugation at 14,000 rpm at 4 °C for 20 min, dissolved in 1× 10 mM Tris-Cl (pH 8.0) and 1 mM EDTA, and subjected to PCR analysis. The results were calculated from three independent real-time PCR experiments and presented as mean ± S.E. of the relative fold enrichment as the percentage of the input signal. The primers for real-time PCR will be provided upon request.

Statistical Analysis—Data are presented as mean ± S.E. of three independent experiments. Two compared groups were analyzed by two-tailed Student’s t test. p < 0.05 (*) and p < 0.01 (**) were considered statistically significant.

RESULTS

The Effects of TNFα on SMRT and β-TrCP1 Protein Accumulation—Through microarray gene expression analyses, we have genes identified previously whose expression is up-regulated by TNFα in HUVECs (24). Because SMRT is an essential corepressor for silencing a broad set of inflammatory response genes in macrophages (7, 8), we hypothesize that SMRT may also play a role in the TNFα-mediated induction of inflammatory genes in HUVECs. To test this, we transiently knocked down SMRT in HUVECs by two independent siRNAs and determined the expression levels of several TNFα target genes. We found that depletion of SMRT in HUVECs significantly enhanced the expression of inflammation-associated genes, including IL-1β, IL-6, and IL-8 (Fig. 1A), suggesting that these TNFα-inducible genes are repressed by SMRT in the absence of TNFα. To test whether TNFα treatment had effects on SMRT protein accumulation, HUVECs were treated with TNFα and harvested at different time points. We found that SMRT protein levels were significantly down-regulated during 4 h of TNFα treatment (Fig. 1B). However, SMRT mRNA levels were unchanged (data not shown). Because TNFα treatment decreases SMRT protein levels but not mRNA, we hypothesize that TNFα regulates SMRT levels through posttranscriptional regulation. Recent reports and our prior observations implied that SMRT can be regulated through ubiquitin-dependent proteolysis (19, 20, 25). It has been shown previously that TNFα induces an E ubiquitin ligase subunit, β-TrCP1, at both the mRNA and protein levels in HEK-293 cells (26), although the role of β-TrCP1 in ECs is completely unknown. Therefore, we examined the correlation between β-TrCP1 and SMRT protein levels in response to TNFα treatment in HUVECs. In contrast to the decrease in SMRT protein levels, β-TrCP1 protein abundance was up-regulated in 4 h of TNFα treatment (Fig. 1B). This observation prompted us to question whether β-TrCP1 affects SMRT protein stability. To test this, we carried out siRNA knockdown experiments to determine the effect of β-TrCP1 knockdown on SMRT protein stability. siRNA-transfected HUVECs were treated with cyclohexamide, and the half-life of endogenous SMRT protein was measured. We found that
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**FIGURE 1. The effects of SMRT knockdown on expression of TNFα-inducible genes.** A, a non-targeting siRNA (siCtrl) or two siRNAs (siS-1 and siS-2) against different exons of SMRT were transiently transfected into HUVECs. After 72 h, cells were harvested, and the mRNA levels of the indicated TNFα-inducible genes were determined by quantitative real-time PCR. *p < 0.05; **p < 0.01. B, HUVECs were treated with 20 ng/ml TNFα and harvested at the indicated times. Aliquots of cells were used to prepare whole cell lysates. Western blot analyses were performed to detect endogenous SMRT protein accumulation. The SMRT and β-TrCP1 protein levels at each time point were quantified by ImageJ and normalized with β-actin. The relative protein levels were further normalized to that at 0 h of TNFα treatment. Changes in the SMRT and β-TrCP1 protein levels in three individual experiments were averaged and plotted as shown in the *right graph*. Total RNA isolation and quantitative real-time PCR were carried out as described under “Experimental Procedures.” C, two siRNAs against β-TrCP1 (siβ-T1-1 and siβ-T1-2) or a non-targeting siRNA were transiently transfected into HUVECs. After 64 h, an aliquot of cells was harvested to examine knockdown efficiency (top left panel). Other aliquots of cells were treated with 50 μM protein translation inhibitor cyclohexamide (CHX). At the time points indicated, cells were collected, and SMRT and β-TrCP1 protein levels were examined by Western blot analyses (bottom panel), quantified by ImageJ, and normalized with β-actin levels. The relative SMRT protein levels at each time point were further normalized to 0 h cyclohexamide treatment, and the trends of SMRT half-life were averaged from three individual experiments and plotted in the associated graph (top right panel).

knockdown of β-TrCP1 prolonged the half-life of endogenous SMRT protein from ~1 h to 4 h (Fig. 1C).

**β-TrCP1-mediated Down-regulation of SMRT Protein**—We further addressed whether β-TrCP1-mediated down-regulation of SMRT can be observed in other cell types and found that knockdown of β-TrCP1 by siRNAs up-regulates SMRT protein levels in HeLa cells (Fig. 2A) without affecting SMRT mRNA accumulation (data not shown). To determine whether β-TrCP1 interacts with SMRT, we carried out GST pull-down assays with several SMRT fragments and found that β-TrCP1 binds to two regions of SMRT, with SMRT (1188–1833) binding more robustly than SMRT (1833–2507) (Fig. 2B). The N terminus fragment, SMRT (1–1194), does not bind GST β-TrCP1. Therefore, we focused our study on amino acids 1188–1833. β-TrCP1 has been identified previously as a substrate-conferring subunit of the Skp1-Cul1-F-box (SCF) ubiquitin E3 ligase complex (27, 28). Through a conserved F box motif, β-TrCP1 binds to Skp1 and Skp1-associated Cul1, Rbx1, and the E2 ubiquitin-conjugating enzyme to assemble a functional E2–E3 supercomplex for ubiquitin transfer (28, 29). The mutant β-TrCP1 (ΔF), which is missing a 45-amino acid F-box motif, had a dominant-negative effect on the degradation of its substrates, CD4 and β-catenin (27, 30). To determine the effect of the F box on the ability of β-TrCP1 to regulate SMRT protein abundance, we tested whether an F box deletion mutant, FLAG-β-TrCP1 (ΔF), was capable of affecting SMRT protein levels (Fig. 2C, *left panel*). Coexpression of wild-type FLAG-β-TrCP1 significantly decreased protein abundance of HA-SMRT (1188–1833). In contrast, FLAG-β-TrCP1 (ΔF) did not have an effect on HA-SMRT (1188–1833) abundance (Fig. 2C, *right panel*). In humans, β-TrCP1 and β-TrCP2 are encoded by two genes that are very similar (Fig. 2C, *left panel*) (26, 31, 32). They share identical amino acid sequences in their C termini but diverge in their N-terminal 100 amino acids. We found that overexpression of β-TrCP1 but not β-TrCP2 down-regulated SMRT protein accumulation in a dose-dependent manner (Fig. 2D). We further demonstrated that FLAG-β-TrCP1-transfected HeLa cells exhibited significantly lower levels of endogenous SMRT, whereas FLAG-β-TrCP1ΔF- or FLAG-β-TrCP2-transfected cells showed similar levels of endogenous SMRT to those in untransfected cells (Fig. 2E). Taken together, these data indicate that β-TrCP1 negatively regulates SMRT protein accumulation.

Previously, we identified a phosphorylation-dependent mechanism underlying Pin1- and Cdk2-mediated down-regulation of SMRT protein levels (20). To determine whether Cdk2 or Pin1 plays a role in β-TrCP1-mediated proteolysis, HeLa cells were cotransfected with FLAG-β-TrCP1 and a Cdk2- and Pin1-resistant mutant, SMRT (3X), in which the Cdk2 phosphorylation sites/Pin1 binding sites were mutated. Interestingly, FLAG-β-TrCP1 was still capable of significantly decreasing SMRT (3X) protein levels (data not shown). These data indicate that the E3 ubiquitin ligase β-TrCP1 down-regulates SMRT protein accumulation independently of the Cdk2-Pin1 pathway.

The Interaction between β-TrCP1 and SMRT—To further dissect the mechanism by which β-TrCP1 down-regulates SMRT, we asked whether β-TrCP1 and SMRT interact in mammalian cells. Fig. 3A demonstrates that anti-SMRT antibodies co precipitated endogenous β-TrCP1 in extracts prepared from HEK-293T cells. Furthermore, immunoprecipitation indicated that HA-SMRT (1188–1833) and FLAG-β-
TrCP1 coprecipitated in HeLa cell extracts (Fig. 3B). β-TrCP1 is known to target phosphorylated substrates (28, 33). To test whether β-TrCP1 binds to SMRT (1188–1833) in a phosphorylation-dependent manner, we first examined whether β-TrCP1 binds HA-SMRT (1188–1833) is phosphorylated. Whole cell extracts were prepared from HA-SMRT (1188–1833)-transfected cells followed by incubation with immobilized purified GST-β-TrCP1 and subsequent treatment with CIP. We found that, after CIP treatment, GST-β-TrCP1-bound HA-SMRT exhibited a slightly increased mobility compared with the untreated sample (Fig. 3C), implying that GST-β-TrCP1-bound HA-SMRT was phosphorylated. We further determined whether phosphorylation of SMRT is required for GST-β-TrCP1 binding. Using CIP-treated cell extracts for pull-down assays, we found that CIP-treated HA-SMRT (1188–1833) was still capable of binding GST-β-TrCP1 (Fig. 3C). These data indicate that the interaction between SMRT and β-TrCP1 is independent of SMRT phosphorylation. Using a reverse GST pull-down assay with GST-SMRT, we were able to confirm that bacterially purified GST-SMRT (1188–1470) interacts with β-TrCP1 in vitro (Fig. 3D). However, similar experiments with another fragment, SMRT (1662–1833), indicated that, in this region, some posttranslational modification was necessary for β-TrCP1 binding. Only mammalian
cell expressed HA-SMRT (1662–1833) and not bacterially expressed GST-SMRT (1571–1833) or GST-SMRT (1672–1833) bound to β-TrCP1 in vitro (Fig. 3, D and E). Because SMRT phosphorylation is not essential for general β-TrCP1 binding, we did not pursue this further.

β-TrCP1 Promotes SMRT Ubiquitination and TNFα-induced SMRT Proteolysis—The function of β-TrCP1/2 is to bridge their substrates to the SCF ubiquitin complex for ubiquitination-mediated proteolysis (28, 29). Through associations with E1 ubiquitin-activating and E2 ubiquitin-conjugating enzymes, the SCF complex promotes ubiquitin transfer from E2 to β-TrCP1/2 substrates (28). To determine whether β-TrCP1 promotes SMRT ubiquitination, we perform in vivo ubiquitination assays. HeLa cells were transiently transfected with FLAG-SMRT (1188–1833) and HA-ubiquitin with or without Myc-β-TrCP1 or the dominant-negative mutant, Myc-
\( \beta \text{-TrCP1} \) (AF box). After blocking proteasome activity with MG132 for 5 h, whole cell extracts were prepared, followed by immunoprecipitation with anti-FLAG antibody and Western blotting with anti-HA antibody. We found that FLAG-SMRT exhibits a higher level of ubiquitination in cells ectopically expressing exogenous Myc-\( \beta \text{-TrCP1} \) than cells without Myc-\( \beta \text{-TrCP1} \) (Fig. 4A, lanes 8 and 9). Conversely, overexpression of the dominant-negative mutant Myc-\( \beta \text{-TrCP1} \) (AF box) significantly decreased FLAG-SMRT ubiquitination (lane 10). The ubiquitin-conjugating enzyme E2D2, one of the associated E2 components (33), was further tested for its role in SMRT ubiquitination. We found that \( \beta \text{-TrCP1} \)-mediated FLAG-SMRT ubiquitination was significantly reduced when the cells were cotransfected with a dominant-negative form of E2D2 (lanes 8 versus lane 11 and lane 9 versus lane 12).

In Figs. 1 and 2, we observed that both TNF\( \alpha \) and \( \beta \text{-TrCP1} \) were capable of down-regulating SMRT protein levels and that TNF\( \alpha \) induces \( \beta \text{-TrCP1} \) protein accumulation. We next asked whether \( \beta \text{-TrCP1} \) plays a role in TNF\( \alpha \)-induced SMRT degradation. To test this, we performed immunoprecipitation of endogenous proteins and found that TNF\( \alpha \) promotes the interaction between \( \beta \text{-TrCP1} \) and SMRT in HUVECs after 2 h of treatment (Fig. 4B). To further determine the role of \( \beta \text{-TrCP1} \) in TNF\( \alpha \)-induced SMRT degradation, we knocked down \( \beta \text{-TrCP1} \), determined the SMRT protein half-life in the presence of TNF\( \alpha \), and found that knockdown of \( \beta \text{-TrCP1} \) significantly prolonged TNF\( \alpha \)-induced SMRT protein half-life (Fig. 4C). Furthermore, TNF\( \alpha \)-induced SMRT degradation was rescued by the addition of MG132, a proteasome inhibitor (Fig. 4D). Moreover, TNF\( \alpha \) treatment increased SMRT ubiquitination (Fig. 4E), and this TNF\( \alpha \)-mediated SMRT ubiquitination was blocked in \( \beta \text{-TrCP1} \) knockdown cells (F). Together, our results support a model in which \( \beta \text{-TrCP1} \) mediates TNF\( \alpha \)-induced ubiquitination- and proteasome-dependent degradation of SMRT.

It has been proposed previously that \( \beta \text{-TrCP1} \) and \( \beta \text{-TrCP2} \) share a redundant function in promoting the TNF\( \alpha \)-induced degradation of IkB\( \alpha \) and subsequent nuclear translocation of p65 (34–37). Consistent with these reports, knockdown of \( \beta \text{-TrCP1} \) alone in HUVECs did not have effects on TNF\( \alpha \)-induced IkB\( \alpha \) degradation (Fig. 4G) nor p65 nuclear localization (H).

The Function of the \( \beta \text{-TrCP1}-\text{SMRT Axis} \) in the Regulation of TNF\( \alpha \) Target Genes—Our observations that TNF\( \alpha \) treatment in HUVECs increases \( \beta \text{-TrCP1} \) and reduces SMRT protein levels suggest that the regulation of TNF\( \alpha \)-inducible genes, in part, is through the \( \beta \text{-TrCP1}-\text{SMRT} \) axis. To further test this hypothesis, we knocked down \( \beta \text{-TrCP1} \) in HUVECs. We observed higher SMRT protein levels and lower expression of TNF\( \alpha \)-inducible genes, such as IL-1\( \beta \), IL-6, and IL-8. These genes are induced in SMRT knockdown ECs and repressed in \( \beta \text{-TrCP1} \) knockdown cells (Figs. 1A and 5A). On the basis of these data, we predict that \( \beta \text{-TrCP1} \) knockdown increases the steady-state levels of SMRT, increases the occupancy of SMRT on the promoters of TNF\( \alpha \) target genes, and, consequently, decreases the expression of TNF\( \alpha \) target genes. Indeed, using ChIP assays, we demonstrate that knockdown of \( \beta \text{-TrCP1} \) leads to increased binding of SMRT to the IL-1\( \beta \), IL-6, and IL-8 promoters but that the binding of SMRT-associated transcription factors, NF-\( \kappa \)B (p50) and BCL-6 (8, 9), was not altered on the IL-1\( \beta \) and IL-8 promoters (Fig. 5B). On the IL-6 promoter, we found that NF-\( \kappa \)B (p50) occupancy increased but that BCL-6 did not bind to this region. Furthermore, the inflammatory gene repression because of \( \beta \text{-TrCP1} \) knockdown was rescued by knocking down SMRT (Fig. 5C), indicating that these TNF\( \alpha \) target genes are regulated by the \( \beta \text{-TrCP1}-\text{SMRT} \) axis. In conclusion, our results provide evidence supporting the hypothesis that TNF\( \alpha \) induces a subset of inflammatory response gene expressions, in part, by elevating \( \beta \text{-TrCP1} \) and, subsequently, down-regulating SMRT protein accumulation (Fig. 6).

DISCUSSION

In this study, we show that TNF\( \alpha \) up-regulates \( \beta \text{-TrCP1} \) protein levels and down-regulates SMRT protein abundance. The latter is caused by \( \beta \text{-TrCP1} \)-mediated posttranslational modification. The loss of SMRT by siRNA knockdown significantly increases expression of TNF\( \alpha \) target genes. Conversely, knockdown of \( \beta \text{-TrCP1} \) results in an extended SMRT half-life, increased SMRT protein levels, increased occupancy of SMRT on its target gene promoters, and decreased TNF\( \alpha \) target gene expression. We have also mapped the \( \beta \text{-TrCP1} \) interacting domain in SMRT and demonstrated that this interaction does not require phosphorylation of SMRT. Taken together, our study provides evidence supporting a model in which \( \beta \text{-TrCP1} \)-dependent SMRT ubiquitination and proteolysis contributes to TNF\( \alpha \)-mediated induction of inflammation-associated genes (Fig. 6).

TNF\( \alpha \)-mediated SMRT Regulation and Acute Inflammation—TNF\( \alpha \) is an immediate-response cytokine secreted by macrophages and the endothelium during acute inflammation (5, 6). A body of studies have elucidated the role of TNF\( \alpha \) in inflammatory responses (38), but how it precisely controls inflammatory gene expression is still not completely understood. Activation of NF-\( \kappa \)B by TNF\( \alpha \) is a major pathway that controls inflammatory gene expression, although it has been shown to be biphasic and depend on the status of its associated coregulators (11–15, 39). On the basis of our findings, the reduction of SMRT protein levels by TNF\( \alpha \) downstream \( \beta \text{-TrCP1} \) E3 ligase may partly account for the activation of NF-\( \kappa \)B activity on the expression of its target genes in HUVECs. Upon TNF\( \alpha \) stimulation, an increase in \( \beta \text{-TrCP1} \) protein level was observed, and this increase was accompanied by an enhanced interaction between \( \beta \text{-TrCP1} \) and SMRT (Figs. 1B and 4B). These observations suggest that an increase in \( \beta \text{-TrCP1} \) plays a role in TNF\( \alpha \)-induced SMRT degradation. Alternatively, but not exclusively, TNF\( \alpha \) may enhance \( \beta \text{-TrCP1} \) and SMRT interaction through a mechanism yet to be elucidated, and this enhanced interaction facilitates \( \beta \text{-TrCP1} \)-mediated SMRT degradation.

SMRT and Inflammation Regulation—In addition to the acute inflammatory response, precise and timely repression of inflammatory genes is equally important to maintain homeostasis in the hematopoietic and immune systems. As a transcriptional corepressor, SMRT integrates diverse signals and cooperates with several transcription factors or repressors, including peroxisome proliferator-activated receptor \( \beta/\delta \), liver X receptor, and BCL-6, to establish a transcriptional repression
$\beta$-TrCP1 and Ubiquitination-mediated SMRT Protein Degradation

A

| FLAG-SMRT | + + + + + + + + + + + |
| HA | - + + + + + + + + + + |
| Myc-$\beta$-TrCP1 | - + + + + + + + + + + |
| Myc-$\beta$-TrCP1 | - + + + + + + + + + + |
| Myc-$\beta$-TrCP1 (dn) | - + + + + + + + + + + |
| Myc-$\beta$-TrCP1 (dn/UBE2D2) | - + + + + + + + + + + |

$\alpha$-FLAG

$\alpha$-Myc

$\alpha$-Myc

$\alpha$-HA

$\alpha$-GFP

WCE

B

| Input | - + - |
| IP: $\alpha$-SMRT | 1 2 3 4 |

| TNF$\alpha$ (20 ng/ml) (2 hr) | $\alpha$-SMRT | $\alpha$-TrCP1 |

C

| siCtrl | si$i\beta$-TrCP1 | si$i\beta$-TrCP1 |
| TNF$\alpha$ (20 ng/ml) (0 hr) | + + + + + + + + + + + |
| CHX (hr) | 0 1 4 |

$\alpha$-SMRT

$\alpha$-TrCP1

$\alpha$-actin

[SMRT] / [Actin]

1.0 0.6 0.2

1.0 1.2 1.1

1.0 0.9 0.7

D

| Input | - + - |
| IP: $\alpha$-SMRT | 1 2 3 |

| TNF$\alpha$ + MG132 | $\alpha$-SMRT |

| $\alpha$-actin |

1 2 3

E

| Input | - + - |
| IP: $\alpha$-SMRT | 1 2 3 |

| MG132 + TNF$\alpha$ + MG132 |

| $\alpha$-Ub |

1 2

F

| siCtrl | si$i\beta$-TrCP1 |
| TNF$\alpha$ (20 ng/ml) (hr) | + + |

| $\alpha$-Ub |

1 2

| $\alpha$-SMRT |

1 2

G

| siCtrl | si$i\beta$-TrCP1 | si$i\beta$-TrCP1 |
| TNF$\alpha$ (20 ng/ml) (10 min - 30 min - 1 h) | + + + |

| $\alpha$-TrCP1 |

1 2 3

| $\alpha$-IKB$\alpha$ |

1 2 3

| $\alpha$-actin |

1 2 3

H

- TNF$\alpha$

a b c d e f g h i j k l m n o p

+ TNF$\alpha$

a b c d e f g h i j k l m n o p

siCtrl si$i\beta$-TrCP1
Whole cell lysates were prepared for coimmunoprecipitation (IP) and normalized with get genes that are induced by LPS, INF-γ, or two individual immunoprecipitated, followed by Western blotting with anti-SMRT and anti-Ub antibodies. A AUGUST 30, 2013 • VOLUME 288 • NUMBER 35 β-TrCP1 and Ubiquitination-mediated SMRT Protein Degradation network (8–10, 39–41). Using a ChIP-sequencing approach, Ghisletti et al. (7) identified a large set of inflammatory genes, including IL-1β, IL-6, and IL-8 as NCoR-SMRT-dependent target genes that are induced by LPS, INF-γ, or phorbol ester. Similarly, IL-1β and IL-6 are also transcriptional targets mutually controlled by repressive BCL-6- and active NF-κB-associated transcriptional complexes in macrophages (9). In this reciprocal trans-regulation system in macrophages, recruitment of NCoR and SMRT to targeted promoters is critical to establish BCL-6-mediated gene repression (8). Notably, the
Inflammation have been observed in obese adipose tissue (18). Diabetes, lower SMRT levels and concomitant low-grade inflammation, in some chronic morbid conditions, such as obesity and chronic diseases, contribute to chronic cardiovascular diseases. Exploring whether deregulation of SMRT in the endothelium promotes ubiquitination-mediated proteasome degradation of SMRT via β-TrCP1-associated SCF E3 ubiquitin ligase, and, hence, elevates the expression of a subset of SMRT and TNFα target genes.

In this study, we also found that knockdown of SMRT in the absence of TNFα is sufficient to up-regulate the expression of inflammatory response genes (Fig. 1A), indicating a pivotal role of SMRT in silencing inflammation in human ECs. Interestingly, in some chronic morbid conditions, such as obesity and diabetes, lower SMRT levels and concomitant low-grade inflammation have been observed in obese adipose tissue (18). Furthermore, deregulation of inflammatory repression and release of inflammatory components such as IL-6, IL-8, or TNFα into the circulation have been proposed to exacerbate chronic diseases and cause further complications (42–44). Following this line, deregulation of SMRT protein levels and low-grade inflammation may also occur in the endothelium under chronic morbid conditions. In the future, it will be worth exploring whether deregulation of SMRT in the endothelium contributes to chronic cardiovascular diseases.

The Role of β-TrCP1 in SMRT Regulation—As a corepressor, SMRT is brought to gene promoters by association with transcription factors to silence target genes. Clearance of SMRT occupancy on target gene promoters is thought to attenuate SMRT-mediated repression and activate gene expression (3, 39). Several mechanisms have been proposed to facilitate SMRT clearance from binding to chromatin. For example, TBL1- and TBLR1-mediated degradation clears SMRT at a subset of NF-κB, AP-1, and nuclear receptor-targeted promoters (45). In addition, early reports indicate that SMRT dissociates from transcription factors such as NF-κB- or thyroid hormone receptor-targeted promoters through signal-induced phosphorylation of SMRT by IKKα, calcium/calmodulin-dependent protein kinase IV, or MEK-1 (16, 46, 47). Our study provides a previously unappreciated mechanism in which β-TrCP1-dependent and ubiquitin-mediated proteolysis contributes to the clearance of SMRT from target gene promoters in response to TNFα stimulation. It has been suggested previously that the β-TrCP family proteins promote ubiquitination and subsequent proteasome-mediated degradation of the inhibitors of NF-κB, IκBα, and IκBβ (35, 36, 48). Our observation that knockdown of β-TrCP1 decreases TNFα target gene expression could theoretically result from its blockade of IκB degradation and subsequent NF-κB p65 nuclear translocation. However, we found that knockdown of β-TrCP1 did not alter IκBα oscillation patterns nor TNFα-mediated nuclear translocation of p65 in HUVECs (Fig. 4, G and H). This observation recapitulates a similar scenario in β-TrCP1−/− mouse embryonic fibroblast cells and β-TrCP1 and β-TrCP2 harbor a conserved functional module, the F-box domain, that bridges substrates to the functional E3 ubiquitin SCF complex (28, 29). Consistent with this notion, deletion of the F box in β-TrCP1 abrogates its ability to down-regulate SMRT protein accumulation. A body of literature indicates that the ability of β-TrCP1 to promote degradation largely relies on its phosphorylation-dependent association with its substrates (28). To our surprise, our data indicate that SMRT is capable of binding β-TrCP1 independently of this posttranslational modification (Fig. 3). This observation provides a rare exception for the interaction between β-TrCP1 and its substrates.

In conclusion, the evidence shown in this study supports a model in which the TNFα downstream ubiquitin E3 ligase β-TrCP1 ubiquitinates the corepressor SMRT, contributing to the derepression of SMRT-targeted proinflammatory genes (Fig. 6). On the basis of these findings, we hypothesize that β-TrCP1 could be a potential target for the development of therapeutic agents for the treatment of inflammation-associated diseases.

Acknowledgments—We thank Dr. David Samols for comments on the manuscript. We also thank Dr. Yu-Ting Su for the anti-ubiquitin antibodies.

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