p53 Protein Regulates Hsp90 ATPase Activity and Thereby Wnt Signaling by Modulating Aha1 Expression*

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Sachiyo Okayama‡, Levy Kopelovich‡, Gabriel Balmus§, Robert S. Weiss*, Brittney- Shea Herbert‡, Andrew J. Dannenberg‡, and Kotha Subbaramaiah*‡

From the †Department of Medicine, Weill Cornell Medical College, New York, New York 10065, the ‡Department of Biomedical Sciences, Cornell University, Ithaca, New York 14853, and the §Department of Medical and Molecular Genetics, Indiana University Simon Cancer Center, Indiana University School of Medicine, Indianapolis, Indiana 46202

Background: The mechanism by which p53, a tumor suppressor gene, regulates Wnt signaling is incompletely understood.

Results: p53 modulates Hsp90 ATPase activity via effects on Aha1 leading to changes in the expression of Wnt target genes.

Conclusion: p53 regulates Hsp90 ATPase activity and thereby Wnt signaling.

Significance: We describe a new mechanism by which p53 affects Wnt signaling.

This article has been withdrawn by the authors. In Fig. 1D, the first lane of the p53 immunoblot was reused as actin in the same figure panel. In Fig. 2B, lanes 1 and 2 of the actin immunoblot were reused in lanes 5 and 6. Lane 2 of the actin immunoblot in Fig. 4K was reused in lanes 3 and 4. The HOP immunoblot in Fig. 4J was reused in Fig. 4 (K and L) as actin. The actin immunoblot in Fig. 4H was reused in Fig. 4 (L and I) as actin. The actin immunoblot in Fig. 5A was reused in Fig. 5B. In Fig. 5H, the c-Myc and Naked-1 immunoblot are the same. There are uncontrolled gel splices in Figs. 5F, 7C, 7F, and 8I. A portion of the actin immunoblot in Fig. 10A was reused in Fig. 10B as Aha1.

The p53 tumor suppressor gene encodes a homotetrameric transcription factor which is activated in response to a variety of cellular stresses. p53 has mechanistic functions in cell cycle arrest and apoptotic responses. When activated, p53 induces expression of pro-apoptotic genes and promotes the degradation of prosurvival proteins, resulting in the inhibition of cell proliferation. p53 regulates a wide range of cellular processes, including cell cycle arrest, apoptosis, senescence, development, and DNA repair.

The development and progression of colon cancer are part of a multistep process in which growth control is increasingly impaired. Activation of the Wnt pathway plays a major role in colon cancer initiation. In normal cells, the levels of cytoplasmic β-catenin are controlled by a multiprotein destruction complex that targets β-catenin for degradation by the proteasome. APC is an intracellular protein that is the heterozygous mutation in the majority of sporadic colon carcinomas and is 80% of sporadic colon carcinomas and is a highly conserved tumor suppressor gene. The APC protein interacts with several other proteins, including Axin, GSK3β, and β-catenin, to form the destruction complex that targets β-catenin for degradation.

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The mechanism by which p53 modulates Wnt signaling is incompletely understood. p53 regulates Hsp90 ATPase activity and thereby Wnt signaling. Significance: We describe a new mechanism by which p53 affects Wnt signaling.

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* To whom correspondence should be addressed: Weill Cornell Medical College, 525 E. 68th St., New York, NY 10065. Tel.: 212-746-4402; Fax: 212-746-4885; E-mail: ksubba@med.cornell.edu.

The abbreviations used are: APC, adenomatous polyposis coli; GSK3β, glycogen synthase 3β; 17-AAG, 17-allylamino-17-demethoxygeldanamycin; Aha1, activator of Hsp90 ATPase; HSF-1, heat shock factor-1; Hsp, heat shock protein; LFS, Li-Fraumeni syndrome; TCF/LEF, T cell factor/lymphocyte enhancer-binding factor.
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link between p53 and Wnt signaling is established, the interaction between these pathways is incompletely understood.

Here we have investigated the effect of p53 on Wnt signaling in human colorectal cancer cell lines and LFS-derived epithelial cells. To translate these in vitro findings, the effect of p53 on Wnt-signaling including target gene expression was also compared in wild type versus p53-null mice. Using these complementary model systems, we show that p53 modulates Wnt signaling via effects on Hsp90. Specifically, loss of p53 function was associated with increased levels of Aha1, a co-chaperone of Hsp90. The changes in Aha1 levels were mediated by HSF-1. Increased interaction of Aha1 and Hsp90 led to enhanced Hsp90 ATPase activity, which stimulated the Akt/GSK3

was associated with increased levels of Aha1, a co-chaperone of Hsp90. The changes in Aha1 levels were mediated by HSF-1. Increased interaction of Aha1 and Hsp90 led to enhanced Hsp90 ATPase activity, which stimulated the Akt/GSK3β pathway. This led, in turn, to increased nuclear translocation of β-catenin and enhanced Wnt target gene expression. Consistent with these findings, we also show that pharmacologic intervention with CP-31398, a p53 rescue compound (16), inhibited the Aha1/Hsp90 axis and thereby suppressed Wnt signaling. Taken together, this study provides new insights into the mechanism by which p53 regulates Wnt signaling, which may be important for understanding the progression of colon cancer.

EXPERIMENTAL PROCEDURES

Materials—CP-31398 was provided by the National Cancer Institute Chemopreventive Agent Repository. Zinc chloride, dimethyl sulfoxide, G418, phosphoenolpyruvate, kinase, NADH, and antibodies to β-actin, HSF-1, and XAP-2 were purchased from Sigma. 17-AAG and methoxygeldanamycin (17-AAG) and hT3R2 were purchased from Cayman Chemicals. PU-H71 was provided by Dr. Arnold J. Levine (Princeton University) (17, 18). These cells were maintained in RPMI 1640 supplemented with 10% FBS and supplemented with 0.5% glutamine (G418). The HME32, HME50, and IUSM/LFS/HME cells were described previously and were provided by Dr. Brittnney-Shea Herbert (Indiana University School of Medicine) (14, 19).

Immunoprecipitation—This assay was performed with a kit from Upstate Biotechnology according to the manufacturer’s instructions. 500–1000 µg of cell lysate or tissue lysate protein was used for immunoprecipitation at room temperature. The immunoprecipitates were then analyzed by Western blotting.

Western Blotting—Cell and tissue lysates were prepared using a lysis buffer (150 mM NaCl, 100 mM Tris, pH 8.0, 1% Tween 20, 50 mM diethylidithiocarbamate, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 µg/ml aprotinin, 10 µg/ml trypsin inhibitor, and 10 µg/ml leupeptin) followed by sonication to remove particulate material. The protein concentration was determined according to Lowry et al. (20). SDS-PAGE was performed under reducing conditions on 10% polyacrylamide gels as described (21). Resolved proteins were transferred onto nitrocellulose sheets and incubated with the indicated antibodies followed by a secondary antibody to horseradish peroxidase-conjugated IgG. The blots were then reacted with the ECL Western blot detection system.

Quantitative Real-time PCR—Total RNA was isolated from colon tissues using the RNeasy mini kit (Qiagen). For tissue analyses, poly(A) RNA was prepared with an Oligotex mRNA mini kit (Qiagen). Poly(A)+ RNA was reversed-transcribed using murine leukemia virus reverse transcriptase and oligo(dT)16 primer. The resulting cDNA was then used for amplification. Primers were designed with an endogenous normalization control from the p53 luciferase plasmid. Real-time PCR was performed using a PerkinElmer ABI 7000 or 7500 Real-time PCR system. The primers used to clone Aha1 promoter were: forward, 5′-GTGAGGGCAGAAACAGAGC-3′; reverse, 5′-CTGCAAAGCAGAAACAGAGC-3′. PCR products of 5′-flanking fragments of Aha1 gene were inserted into the KpnI site of the luciferase basic plasmid vector, pGL2 (Promega). The subcloned PCR products were sequenced by using T7 and SP6 promoter primers to confirm that the products were the authentic promoter fragments. Site-directed mutagenesis was used to mutate the HBE (relative quantification)

Cell Culture—HCT15, DLD-1, and LoVo human colon cancer cell lines were obtained from the American Type Culture Collection (ATCC). These cell lines were maintained according to ATCC instructions. The human colon carcinoma cell line EB-1 was kindly provided by Dr. Arnold J. Levine (Princeton University) (17, 18). These cells were maintained in RPMI 1640 medium with 10% FBS and supplemented with 0.5%/lit
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DNA fragments. After centrifugation, the cleared supernatant was incubated with 4 μg of the HSF-1 antibody at 4 °C overnight. Immune complexes were precipitated, washed, and eluted as recommended. DNA-protein cross-links were reversed by heating at 65 °C for 4 h, and the DNA fragments were purified and used as a template for PCR amplification.

Quantitative real-time PCR was carried out. Aha1 promoter oligonucleotide sequences for PCR primers were: forward, 5’-GCAGGGAGGTGCTTATTA-3’ and reverse, 5’-TAGATGGCCACAAAAACG-3’. This primer set encompasses the Aha1 promoter sequence from nucleotide −282 bp to −583 bp. PCR was performed at 94 °C for 30 s, 62 °C for 30 s, and 72 °C for 1 min for 30 cycles.

**FIGURE 1.** *p53 regulates Wnt signaling.* A, C, and E, cells were transfected with 0.45 μg each of TOP Flash and FOP Flash constructs and 0.2 μg of pSVβgal. Cells also received 0.9 μg of siRNA to GFP (control siRNA) or p53. 48 h after transfection, cells were harvested, and luciferase activity was measured. TOP Flash activity was determined by the ratio of pTOP-flash to pFOP-flash luciferase activity, each normalized to β-galactosidase enzymatic activity levels. B, D, and F, cells were transfected with 2 μg of siRNA (control) to GFP or p53 for 48 h. Following transfection, cells were harvested, and cell lysates were subjected to Western blotting. The blots were probed with antibodies to the indicated proteins. G and H, EB-1 cells were transfected with 1.8 μg of p53 luciferase construct (G) or 0.9 μg each of TOP Flash and FOP Flash constructs and 0.2 μg of pSVβgal for 24 h. 24 h later, cells were treated with indicated concentrations of ZnCl2 for 12 h, and then cells were harvested, and luciferase activity was measured. Luciferase activity was normalized to β-galactosidase activity. I, cells were treated with the indicated concentrations of ZnCl2 for 12 h. Cell lysates were subjected to Western blotting, and the blots were probed as indicated. A, C, E, G, and H, mean ± S.D. (error bars) are shown, n = 6. *p < 0.01 compared with control siRNA-treated cells (A, C, and E) or vehicle-treated cells (G and H).
45 s for 35 cycles, and real-time PCR was performed at 95 °C for 15 s and 60 °C for 60 s for 40 cycles. The PCR product generated from the ChIP template was sequenced, and the identity of the Aha1 promoter was confirmed.

Hsp90 ATPase Activity—The ATPase assay was based on a regenerating coupled enzyme assay as described earlier (22, 23). Briefly, Hsp90 was immunoprecipitated from cell lysates, and the pellet was resuspended and used for assay. Reaction was conducted in a 1 ml assay containing 100 mM Tris-HCl, pH 7.4, 20 mM KCl, 6 mM MgCl₂, 0.8 mM ATP, 0.1 mM NADH, 2 mM phosphoenolpyruvate, 0.2 mg of pyruvate kinase, 0.05 mg of lactate dehydrogenase, and Hsp90 immunoprecipitated from cell and tissue lysates. Equal amounts of Hsp90 protein were used in each treatment group. Sufficient NADH was added to give an initial absorbance of 0.3 at 340 nm before addition of Hsp90 and activity was detected as a decrease in absorbance. Hsp90 ATPase activity is expressed as pmol/min per mg of protein.

Animal Model—p53 knock-out mice carrying the Trp53tm1Tyj allele were maintained on the 129S6 inbred strain background and genotyped by PCR analysis of DNA extracted from tail tip biopsies (24). Experimental p53/H11002/H11002 and sex-matched littermate control p53/H11001/H11001 mice at 6–10 weeks of age were euthanized by carbon dioxide asphyxiation, and isolated colon tissue was rinsed once with PBS and then snap frozen for subsequent molecular analysis. All animal use was conducted in accordance with federal and institutional guidelines, under a protocol approved by the Cornell University Institutional Animal Care and Use Committee.

FIGURE 2. CP-31398 inhibits Wnt signaling. A, C, and E, HCT-15 (A), LoVo (C), and DLD-1 (E) cells were transfected with 1.8 μg of p53 luciferase construct and 0.2 μg of p5SV/lac. 24 h later, the cells were treated with the indicated concentrations of CP-31398 for 24 h, and then luciferase activity was measured. Luciferase activity was normalized to β-galactosidase activity. B, D, and F, HCT-15 (B), LoVo (D), and DLD-1 (F) cells were treated with CP-313198 for 24 h, and cell lysates were harvested for Western blot analysis. Immunoblots were probed with antibodies as indicated. A, C, E, mean ± S.D. (error bars) are shown, n = 6. *, p < 0.01 compared with vehicle-treated cells.
Statistics—Comparisons between groups were made by Student’s t test. A difference between groups of $p < 0.05$ was considered significant.

RESULTS

p53 status has been identified as a determinant of Wnt signaling, but the mechanisms are incompletely understood (6–8). To further understand the interaction between p53 and Wnt signaling, both in vitro and animal models were employed.

p53 Regulates Wnt Target Gene Expression—First, we studied the effects of p53 status on Wnt target gene expression, namely Axin-2, c-Myc, and Naked-1. Silencing p53 in HCT-15 (p53$^{−/−}$), DLD-1 (p53$^{−/−}$), and LoVo (p53$^{−/−}$) colon cancer cells led to decreased levels of p21, a downstream target of p53, a marked increase in β-catenin/TCF/LEF transcriptional activity, and enhanced expression of Wnt target proteins (Fig. 1, A–F). The importance of p53 as a determinant of Wnt signaling was further evaluated in EB-1 cells, a p53-null cell line that expresses Zn2$^{+}$-inducible wild type p53 (17, 18). Induction of p53 in EB-1 cells was associated with increased p53 transcriptional activity (Fig. 1 G), decreased β-catenin/TCF/LEF transcriptional activity (Fig. 1 H), and reduced levels of Axin-2, c-Myc, and Naked-1 (Fig. 1 I). In this context, levels of β-catenin decreased whereas TCF4 expression was unaltered (data not shown). Moreover, pharmacological intervention with CP-31398, a p53 rescue/stabilizing compound, caused a dose-dependent increase in p53 levels and transcriptional activity and reduced expression of Wnt target genes (Fig. 2, A–F). These
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results clearly demonstrate that p53 status, whether by silencing or pharmacological rescue, modulates Wnt signaling in human colorectal cancer cell lines.

p53 Status Is a Determinant of Hsp90 ATPase Activity—Hsp90, a chaperone molecule for many client proteins including those with oncogenic potential, is activated in a variety of malignancies (25–27). Here we determined whether p53 status is a determinant of Hsp90 ATPase activity. Silencing p53 caused a significant increase in Hsp90 ATPase activity in HCT-15, LoVo, and DLD-1 cells (Fig. 3, A–C). This increase was observed in the absence of increased Hsp90 protein levels. In contrast, inducing p53 in EB-1 cells (Fig. 3D) or by treatment with CP-31398 (Fig. 3, E and F) led to a dose-dependent inhibition of Hsp90 ATPase activity. This decrease in Hsp90 ATPase activity occurred in the absence of corresponding changes in Hsp90 protein levels. Taken together, these results indicate that p53 is a determinant of Hsp90 ATPase activity in colon cancer cells.

p53 Modulates Hsp90 ATPase Activity and Wnt Target Gene Expression through Aha1—Previously, Hsp90 ATPase activity was found to regulate Wnt signaling (28). Given this background, we tested the effects of two inhibitors of Hsp90 ATPase. Both 17-AAG (29) and PU-H71 (30), prototypic Hsp90 ATPase inhibitors, caused dose-dependent suppression of ATPase. Both 17-AAG (29) and PU-H71 (30), prototypic Hsp90 ATPase inhibitors, caused dose-dependent suppression of ATPase. Both 17-AAG (29) and PU-H71 (30), prototypic Hsp90 ATPase inhibitors, caused dose-dependent suppression of ATPase.

Axin-2, c-Myc, and Naked-1 in HCT15, DLD-1, and EB-1 cells were further evaluated in EB-1 cells. Here, treatment with ZnCl₂, which induced p53 (Fig. 1I), caused a corresponding decrease in Aha1 expression (Fig. 4D). In contrast, silencing Aha1 in HCT-15, LoVo, and DLD-1 cells caused a substantial increase in Aha1 levels (Fig. 4, E–G). Importantly, the effect of p53 on Hsp90 co-chaperones appears to be specific to Aha1 because, in similar experiments, whether through silencing of p53 or its rescue by pharmacologic intervention with CP-31398, levels of other co-chaperones of Hsp90, namely p23, XAP-2, and HOP, were unaffected (Fig. 4, H–M). In addition to HCT-15 cells, levels of co-chaperones other than Aha1 were unaffected in LoVo, DLD-1, and EB-1 cell lines (data not shown).

To test directly the role of Aha1 in regulating Hsp90 ATPase activity, Hsp90 ATPase activity was measured following silencing of Aha1. Notably, silencing of Aha1 led to reduced Hsp90 ATPase activity in HCT-15, EB1 (Fig. 5, A and B), and DLD-1 cells (data not shown). Next, co-immunoprecipitation experiments were conducted to determine whether changes in p53 levels modified the interaction between Hsp90 and Aha1. As shown in Fig. 5, C and D, silencing p53 led to increased interaction between Aha1 and Hsp90. Similar effects were observed in LoVo cells (data not shown). In contrast, treatment with CP-31398 or p53 induction in EB-1 cells led to decreased interaction between Aha1 and Hsp90 (Fig. 5, E and F). To evaluate the functional significance of Aha1 in regulating Wnt target gene expression, we investigated the effects of silencing Aha1. As shown in Fig. 5, G and H, silencing of Aha1 led to reduced levels of Axin-2, c-Myc, and Naked-1. These results indicate that p53 modulates the expression of Aha1 and its interaction with Hsp90 which, in turn, affects Hsp90 ATPase activity and thereby the expression of genes regulated by Wnt signaling.

Next we investigated the mechanism by which p53 regulated levels of Aha1. To determine whether p53 regulated Aha1 transcription, transient transfections were carried out. Silencing of p53 was associated with increased Aha1 promoter activity (Fig. 6, A–C). Treatment with ZnCl₂, which induced p53 (Fig. 1I), caused a corresponding dose- and time-dependent decrease in Aha1 promoter activity (Fig. 6, D and E). Previously, HSF-1 was found to be a potential regulator of Aha1 expression (34). Therefore, we next evaluated whether the p53-dependent effects on Aha1 were mediated by HSF-1. A potential HSF-1-binding element (−331 to −316) was identified in the human Aha1 promoter (Fig. 6F). Transient transfections were carried out using a series of Aha1 promoter deletion constructs. Notably, silencing p53 stimulated Aha1 promoter activity, an effect that was lost when the HSF-1-binding element was deleted or mutated (Fig. 6, G and H). Consistent with these findings, the suppressive effects of wild type p53 were lost when the HSF-1-binding element was deleted or mutated (Fig. 6, I and J). Having established the importance of HSF-1 for regulating Aha1 promoter activity, ChIP assays were performed to evaluate the
binding of HSF-1 to the Aha1 promoter. As shown in Fig. 7 A and B, p53 regulated the binding of HSF-1 to the Aha1 promoter. More specifically, treatment with ZnCl2, which induced p53, suppressed the recruitment of HSF-1 to the Aha1 promoter; silencing of p53 increased the recruitment of HSF-1 to the Aha1 promoter. Moreover, silencing of HSF-1 led to reduced Aha1 protein levels (Fig. 7 C). Given the important effects of HSF-1 on Aha1 levels, we next determined whether changes in HSF-1 levels modulated Hsp90 ATPase activity. Silencing HSF-1 was associated with reduced Hsp90 ATPase activity in both EB-1 and HCT-15 cells (Fig. 7, D and E). Finally, silencing of HSF-1 led to reduced expression of Wnt target genes (Fig. 7 F).

p53 Regulates Akt Phosphorylation—The PI3K/Akt pathway is commonly deregulated in human cancers. Akt is a client protein of Hsp90, which upon activation phosphorylates GSK3β, stabilizes β-catenin, and induces the expression of Wnt target genes (28, 35–38). Accordingly, we determined the effects of LY294002, a PI3K inhibitor, on the expression of Axin-2, c-Myc, and Naked-1. Treatment with LY294002 caused a dose-dependent decrease in levels of Axin-2, c-Myc, and Naked-1 (Fig. 8 A and B). Silencing p53 in these cells induced Akt phosphorylation (data not shown). Induction of p53 in EB-1 cells inhibited Akt phosphorylation (Fig. 8C). Silencing Aha1 inhibited Akt phosphorylation (Fig. 8D). Pharmacologic inhibition of Hsp90 ATPase was also associated with reduced Akt phosphorylation (Fig. 8E). Silencing Akt1 in these cells caused a significant decrease in the levels of Wnt target proteins including Axin-2, c-Myc, and Naked-1 (Fig. 8F). Together, these results support the notion that p53 status regulated the Aha1/Hsp90 axis resulting in changes in Akt activity that led, in turn, to altered expression of Wnt target genes.

p53 Status Is a Determinant of GSK3β Phosphorylation—GSK3β serves a critical role in Wnt signaling during cancer development (1, 2). GSK3β can be phosphorylated by Akt, leading to activation of TCF/LEF-dependent gene expression. Here we investigated whether p53 status could affect GSK3β phosphorylation via the Hsp90/Aha1/Akt axis. Silencing of p53 caused a significant increase in GSK3β phosphorylation, whereas induction of wild type p53 in EB-1 cells or pharmacologic rescue of mutant p53 with CP-31398 caused a substantial reduction of GSK3β phosphorylation (Fig. 8, G–I). The effects of CP-31398 on the Aha1/Akt/GSK3β pathway occurred within four h and persisted for up to 24 h (data not shown). Along similar lines, silencing Aha1 or inhibiting Hsp90 caused a marked reduction in GSK3β phosphorylation (Fig. 8, J and K). These results suggest that p53 regulates the Aha1/Hsp90/Akt/GSK3β axis and thereby the expression of Wnt target genes (Fig. 8L).

Levels of Aha1, Hsp90 ATPase Activity, and Wnt Target Genes Are Increased in p53-null Mice—To assess the impact of p53 on the Aha1/Hsp90 ATPase axis in vivo, we utilized colon...
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A. HCT-15

B. LoVo

C. DLD-1

D. EB-1

E. EB-1

F. HBE

G. -933/+112

H. -450/+112

I. -314/+112

J. HBE Mut.

-933/+112

Control siRNA

p53 siRNA

ZnCl₂ (μM)

-331 -316

-314/+112

Control

ZnCl₂

-933/+112

-450/+112

-314/+112

-933/+112

Control siRNA

p53 siRNA

HBE

WITHDRAWN

December 2, 2019
FIGURE 7. Wnt target gene expression is regulated by HSF-1. A, EB-1 cells were treated with 75 μM ZnCl$_2$ for 4 h. B, HCT-15 cells were transfected with 2 μg of siRNA to GFP (control) or p53 for 48 h. C, cells were transfected with 2 μg of siRNA to GFP (control) or HSF-1 for 48 h. C, cell lysates were subjected to Western blotting, and the blots were probed as indicated. D and E, Hsp90 ATPase activity was measured. Cell lysates were also subjected to Western blotting and the blots probed as indicated (insets). Mean ± S.D. (error bars) are shown, n = 6. *, p < 0.01 compared with control siRNA-treated cells. F, cell lysates were subjected to Western blotting and the blots probed as indicated.

FIGURE 6. HSF-1 is important for p53-mediated regulation of Aha1. A–C, the indicated cells were transfected with 0.9 μg of Aha1 promoter luciferase construct and 0.2 μg of pSVβgal. Cells also received 0.9 μg of siRNA to GFP (control siRNA) or p53 for 48 h after transfection, cells were harvested, and luciferase activity was measured. Luciferase activity was normalized to β-galactosidase activity. D and E, EB-1 cells were transfected with 1.8 μg of Aha1 promoter luciferase construct and 0.2 μg of pSVβgal. Subsequently, cells were treated with the indicated concentrations of ZnCl$_2$ for 12 h (D) or with 100 μM ZnCl$_2$ (E) for the indicated time period. Cells were harvested, and luciferase activity was measured. Luciferase activity was normalized to β-galactosidase activity. F, schematic represents Aha1 promoter deletion constructs that were used. HBE, represents the HSF-1-binding element. G and H, HCT-15 cells were transfected with 0.9 μg of Aha1 promoter deletion luciferase constructs as indicated and 0.2 μg of pSVβgal. Cells also received 0.9 μg of siRNA to GFP (control siRNA) or p53. 48 h after transfection, cells were harvested, and luciferase activity was measured. Luciferase activity was normalized to β-galactosidase activity. I and J, EB-1 cells were transfected with the 1.8 μg of the indicated Aha1 promoter luciferase constructs and 0.2 μg of pSVβgal. Subsequently, cells were treated with 100 μM ZnCl$_2$ for 12 h, and luciferase activity was measured. Luciferase activity was normalized to β-galactosidase activity. A–C, G, and H, mean ± S.D. (error bars) are shown, n = 6. *, p < 0.01 compared with control siRNA-treated cells. D, E, I, and J, mean ± S.D. (error bars) are shown, n = 6. *, p < 0.01 compared with vehicle (control)-treated cells.
tissue from p53 wild type and p53-null mice. Compared with wild type mice, p53-null mice exhibited both increased Hsp90 ATPase activity and elevated Aha1 levels (Fig. 9, A and B). To evaluate the interaction between Hsp90 and Aha1, immunoprecipitation experiments were carried out. As shown in Fig. 9C, the interaction between Hsp90 and Aha1 was markedly increased in p53-null mice. Moreover, Akt and GSK3β phosphorylation were both increased in colon tissues from p53-null mice (Fig. 9, C and D). These changes in LFS-derived, p53 heterozygous epithelial cells appear to recapitulate alterations seen in colon tumor cells.

**DISCUSSION**

In the present study, we have confirmed previous results suggesting that p53 affects Wnt signaling (6–8). Notably, our data demonstrate for the first time that Hsp90 plays a significant role in mediating this effect. Several lines of evidence support this point. Levels of Hsp90 ATPase activity were increased in LFS-derived p53 heterozygous versus normal epithelial cells. Silencing of p53 led to increased Hsp90 ATPase activity in multiple colorectal cancer cell lines. Moreover, expression of wild type p53 in EB-1 cells, a p53-null cell line, led to gene dose-dependent suppression of Hsp90 ATPase activity. Similarly, use of CP-31398, a p53 rescue compound, caused dose-dependent suppression of Hsp90 ATPase activity. These findings showed that 17-AAG and CP-31398, Hsp90 ATPase inhibitors, down-regulated Wnt target genes including Axin-2, c-Myc, and Naked-1 in cell lines.

P53-mediated changes in Hsp90 ATPase activity were found in the absence of altered levels of Hsp90 protein, suggesting that Aha1 was regulated by Hsp90 ATPase activity. The significance of Hsp90 ATPase activity in regulating Aha1 levels leading to altered Hsp90 ATPase activity was investigated using a reporter assay in which p53 regulates Aha1 levels via the HSF-1 element. Aha1 protein levels were associated with corresponding alterations in the interactions between Aha1 and Hsp90 and Hsp90 ATPase activity. To further evaluate the functional significance of Aha1 for mediating p53-dependent changes in Hsp90 ATPase activity, Aha1 was silenced. Silencing of Aha1 inhibited Hsp90 ATPase activity and the expression of Wnt target genes. The mechanism by which p53 regulates Aha1 levels was previously unknown. Aha1 can be regulated by HSF-1 (34). We identified an HSF-1 binding site in the promoter of Aha1. Promoter analyses indicated that p53 regulated Aha1 expression via the HSF-1 element. For example, silencing p53 induced Aha1 promoter activity; this effect was lost when cells were transfected with an Aha1 promoter construct that did not contain the HSF-1 binding site or when the HSF-1 binding site was mutagenized. Additionally, ChIP assays showed increased recruitment of HSF-1 to the Aha1 promoter when p53 was silenced and decreased recruitment when wild type p53 was overexpressed. Moreover, silencing HSF-1 led to reduced Aha1 levels, decreased Hsp90 ATPase, and suppression of Wnt target gene expression. Taken together, these results suggest a novel mechanism by which p53 regulates Hsp90 ATPase activity through Aha1, which is a co-chaperone of Hsp90. This mechanism may be important for the development of colorectal cancer and other diseases.
together, these data suggest that HSF-1 is important for p53-mediated regulation of Wnt signaling. Of potential relevance, silencing HSF-1 has been reported to sensitize cancer cells to Hsp90 inhibition (43).

A link between Hsp90 ATPase activity and Wnt signaling has been reported (28). Akt is a known client protein of Hsp90 (35, 36). Inhibition of the PI3K/Akt signaling pathway can block nuclear localization of β-catenin (28, 37, 38). Here we demonstrate that Hsp90 ATPase activity regulates the phosphorylation of Akt and thereby Wnt target gene expression. Inhibition of Hsp90 ATPase led to reduced levels of phospho-Akt. Consistent with these findings, silencing of Aha1 also reduced Akt activity. Moreover, treatment with LY294002, a PI3K inhibitor or silencing Akt1, inhibited the expression of Axin-2, c-Myc, and Naked-1. Because of the link between p53 and Hsp90 ATPase activity, we also confirmed that Akt activity was p53-dependent. In support of this possibility, overexpressing wild type p53 in colon cancer cells led to decreased Akt phosphorylation. Levels of phospho-Akt were also increased in cells from patients with LFS. Taken together, these data show that increased Hsp90 ATPase activity, associated with either mutation or loss of p53, led to enhanced Akt activity and increased Wnt target gene expression. Akt can phosphorylate GSK3β, resulting in the accumulation of β-catenin (28, 37, 38). Several experiments were performed to evaluate whether this mechanism was operative in a p53-dependent manner. Modulating p53 levels led to changes in GSK3β phosphorylation. Similarly, enhanced GSK3β phosphorylation and increased β-catenin levels were observed in LFS cells. Silencing of Aha1 or inhibiting Hsp90 ATPase activity also suppressed GSK3β phosphorylation. Collectively, these in vitro findings suggest that p53 regulates the Aha1/Hsp90 ATPase axis leading to changes in Akt/GSK3β signaling thereby modulating Wnt target gene expression. Recently, microRNA-34 was found to be important for p53-mediated regulation of Wnt signaling (8). Although Aha1 is not a direct target of microRNA-34, its interacting partner GTP cyclohydrolase-1 is regulated by it (44, 45). Possibly, microRNA-34 will modulate Hsp90 ATPase activity via effects on GTP cyclohydrolase-1. Future studies are needed to determine whether microRNA-34 regulates HSF-1 or Aha1 levels and thereby Hsp90 ATPase activity.
In an attempt to translate the in vitro findings described above, the Aha1/Hsp90/Akt/GSK3β/H9252 axis was evaluated in colon tissues from wild type versus p53-null mice. Consistent with the in vitro results, increased Aha1 levels, Hsp90 ATPase activity, Akt and GSK3β/phosphorylation and Wnt target expression was observed in colons of p53-null compared with p53 wild type mice. Remarkably, immunoprecipitation experiments indicated an increased interaction between Hsp90 and Aha1 in the colons of p53-null mice. These results suggest that colons of p53-null mice have a heightened Hsp90 ATPase activity and increased Wnt signaling. Previously, the p53 rescue compound CP-31398 was found to reduce intestinal tumorigenesis in APCmin/H-11001 mice (46). Additionally, p53 deficiency has been shown to accelerate tumorigenesis in Wnt-1 transgenic mice (15). Our observation that p53 activation suppresses Wnt signaling may help to explain these prior observations. Whether inhibitors of Hsp90 ATPase will have a similar protective effect should be tested. LFS patients are at increased risk for a number of tumor types including colon cancer (47, 48). Our observation that cells from LFS patients had elevated Aha1 levels, Hsp90 ATPase activity, Akt and GSK3β phosphorylation and increased Wnt target gene expression may further help to explain the link between p53 and increased risk of tumor formation. Possibly, agents that disrupt this activated signaling axis will possess chemopreventive properties. Another potential implication of our findings relates to cancer therapy. If p53 status is found to be a determinant of Hsp90 ATPase activity in tumors, this could contribute to chemoresistance or provide a strategy for identifying patients who are most likely to benefit from inhibitors of Aha1 or Hsp90 ATPase activity (34, 49–51).

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