WT1 Induction of Mitogen-Activated Protein Kinase Phosphatase 3 Represents a Novel Mechanism of Growth Suppression

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Abstract

In its role as a tumor suppressor, WT1 transactivates several genes that are regulators of cell growth and differentiation pathways. For instance, WT1 induces the expression of the cell cycle regulator p21, the growth-regulating glycoprotein amphiregulin, the proapoptotic gene *Bak*, and the Ras/mitogen-activated protein kinase (MAPK) inhibitor Sprouty1. Here, we show that WT1 transactivates another important negative regulator of the Ras/MAPK pathway, MAPK phosphatase 3 (MKP3). In a WT1-inducible cell line that exhibits decreased cell growth and increased apoptosis on expression of WT1, microarray analysis showed that *MKP3* is the most highly induced gene. This was confirmed by real-time PCR where *MKP3* and other members of the fibroblast growth factor 8 syn expression group, which includes *Sprouty 1* and the Ets family of transcription factors, were induced rapidly following WT1 expression. WT1 induction was associated with a block in the phosphorylation of extracellular signal-regulated kinase in response to epidermal growth factor stimulation, an effect mediated by MKP3. In the presence of a dominant-negative MKP3, WT1 could no longer block phosphorylation of extracellular signal-regulated kinase. Lastly, when MKP3 expression is down-regulated by short hairpin RNA, WT1 is less able to block Ras-mediated transformation of 3T3 cells.
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Introduction

WT1 encodes a transcription factor required for normal kidney development that is mutated in 10% to 15% of sporadic Wilms' tumors. Constitutional mutation of WT1 has been described in three developmental syndromes, WAGR, Denys-Drash, and Frasier (1), which are associated with genitourinary malformations and Wilms' tumor. Bona fide WT1 target genes, activated or repressed by the protein, are gradually being identified. Much of this work has been carried out by gain-of-function studies in heterologous cell systems such as NIH3T3 fibroblasts (2) or Saos-2 osteosarcoma cells (3). Nevertheless, these studies have yielded WT1 targets validated by their expression in the developing kidney and their roles in growth control. WT1 has both activation and repression domains located in the NH_2 terminus; however, our previous work suggested that the growth suppression activity of WT1 was most closely linked to its ability to induce gene expression (4).

WT1 target genes may be thematically grouped. WT1 can induce the expression of genes implicated in renal development and the mesenchymal-epithelial transition, such as E-cadherin (2), podocalyxin (5), nephrin (6), and Wnt4 (7). Hence, the growth-regulatory role of WT1 may be tightly linked to its role in guiding development. Depending on the cellular context, WT1, through its actions on apoptotic regulators such as Bcl2 (8, 9), Bfi1 (10), Bak, and several BH3-only proteins (11), can increase or decrease apoptosis. And, like the archetypal tumor suppressor p53, WT1 can inhibit cell growth through activation of $p21^{WAF1/CIP1}$ (12), a common feature of differentiating cells. WT1 also regulates signaling by growth factor receptors. WT1 induces the expression of amphiregulin, which is secreted by the metanephric mesenchyme to stimulate the growth of the ureteric tree (3). Similarly, WT1 induces the expression of TrkB, a growth factor receptor critical for coronary vasculature development (13). However, WT1 can also down-regulate growth factor pathways. For example, WT1 represses expression of the insulin-like growth factor-I receptor (14), the epidermal growth factor receptor (EGFR; refs. 15, 16), connective tissue growth factor (17), and vascular endothelial growth factor (18).

We previously reported that *sprouty1* (*Spry1*), an inhibitor of fibroblast growth factor receptor (FGFR) signaling through the Ras/mitogen-activated protein kinase (MAPK) pathway, is a direct target gene of WT1 and is required for normal glomeruli formation (19). Here, we show that the MAPK phosphatase 3 (MKP3), another important inhibitor of the Ras pathway, is also regulated by WT1. MKP3 is a member of the dual specificity phosphatase family that dephosphorylates both serine/threonine and tyrosine residues, leading to the inactivation of MAPKs (20). MKP3 is a highly specific inhibitor of

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extracellular signal-regulated kinase (ERK) 1/2 but has little effect on c-Jun NH_2-terminal kinase/stress-activated protein kinase and p38 MAPKs (21, 22). Like Spry1, MKP3 is detected in the ureter tips of the developing kidney, and in the developing limb buds and lung, mirroring places of high FGF8 expression (23, 24).

Within the cell, MKP3 is localized to the cytoplasm (21) where it binds specifically and tightly to the COOH terminus of ERK1/2 (25) and is engaged in a complicated negative-positive feedback loop with ERK1/2. On growth factor stimulation, ERK1/2 is phosphorylated, translocates to the nucleus, and phosphorylates nuclear targets such as Elk1. MKP3 has a low basal activity, but after ERK2 pathway activation, MKP3 is transcriptionally up-regulated (21, 26) and its enzymatic activity is stimulated (27, 28). Activated MKP3 binds to, dephosphorylates, and sequesters ERK in the cytoplasm in an inactive form. In turn, MKP3 is regulated by ERK2 (27-29). On binding to MKP3, ERK2 phosphorylates MKP3, leading to its proteasomal degradation. In this way, ERK1/2 exerts a positive feedback on its own activity by promoting the degradation of one of its major inactivators (30). MKP3 gene expression in the mouse, *Drosophila, Xenopus*, and zebrafish is localized to areas of high EGFR and FGFR signaling, suggesting that MKP3 constitutes an evolutionarily conserved negative feedback loop on the activity of the Ras/MAPK signaling pathway (21, 31, 32). The fact that WT1 transactivates two negative regulators of the Ras/MAPK pathway suggests that this is a major target of WT1 function.

Results

Wild-Type WT1A Activates Transcription of MKP3

Microarray analysis of Saos-2 cells induced to express WT1A (33) indicated that *MKP3/DUSP6* was a highly induced gene. Real-time PCR analysis confirmed that expression of WT1A for 18, 24, and 48 h led to an increase in expression of *MKP3,* ranging from 12- to 90-fold, whereas WT1A-112, a point mutant of WT1 defective for transcriptional activation, minimally induced *MKP3* expression (3-fold). MKP3 is a member of a syn expression group regulated by FGF8 during limb bud development (34). Members of this group include Sef, a negative regulator of FGF signaling, and the Ets transcription factors ETV1, ETV4, and ETV5. In our system, these Ets genes were significantly induced by WT1, as assessed by real-time PCR (Fig. 1A) and corroborated to varying degrees by microarray analysis (e.g., ETV1: 11.5-fold; ETV5: 13-fold). The *Sef* gene was not represented on the microarray chip; however, Spry1, a relative to Sef, was also significantly induced by WT1 (11).

MKP3 mRNA was induced 3-fold within 6 h of WT1 induction (Fig. 1B), suggesting that it might be a direct transcriptional target of WT1. To test this hypothesis, a *MKP3* promoter reporter plasmid, encompassing two putative WT1-binding regions, was coexpressed in 293T cells with expression vectors for wild-type or mutant WT1A. Wild-type WT1A activated the promoter in a dose-dependent manner, whereas the mutant WT1A-112 did not (Fig. 2B). Chromatin immunoprecipitation from the Wilms' tumor cell line, CCG99-11,

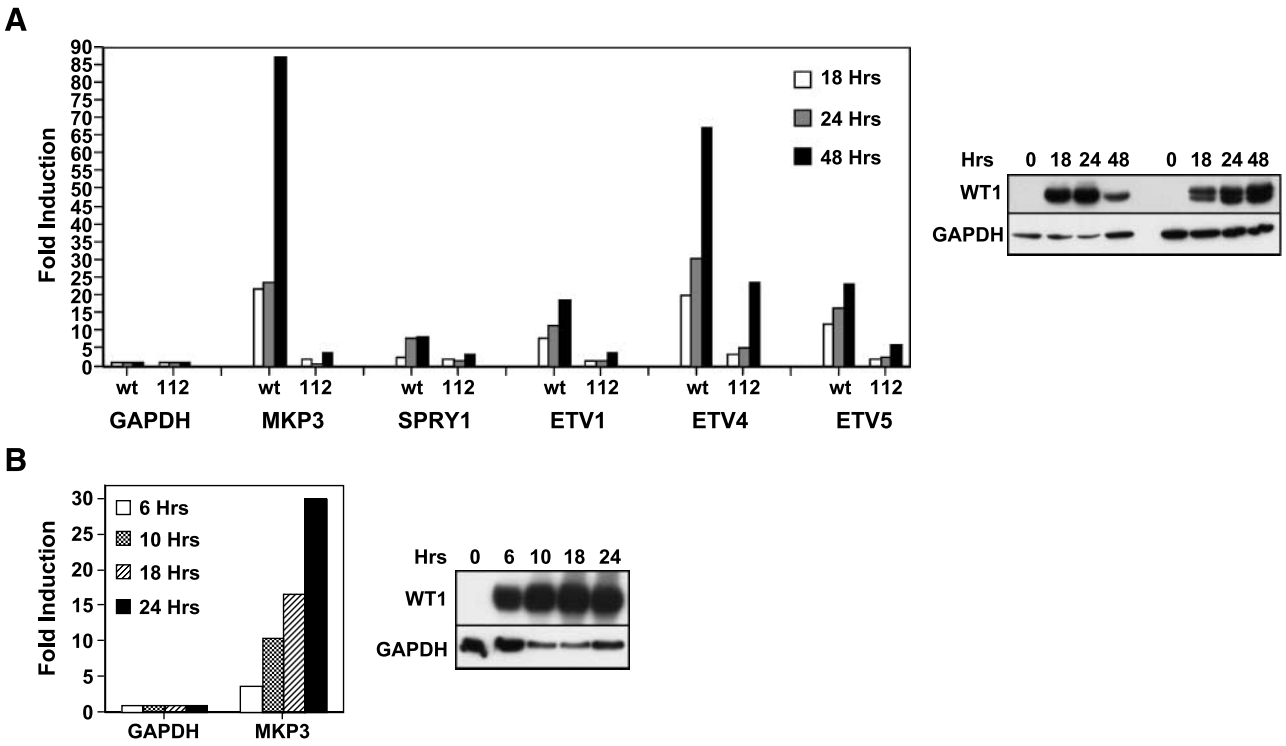

FIGURE 1. Induction of MKP3 and FGF8 syn expression group genes by WT1. **A** and **B.** Real-time PCR analysis of indicated genes in Saos-WT1–inducible cell line following withdrawal of tetracycline. Data were calculated as fold induction relative to pre-WT1 induction condition after normalizing with GAPDH. Western blots corresponding to each induction experiment show expression of induced WT1 over time.

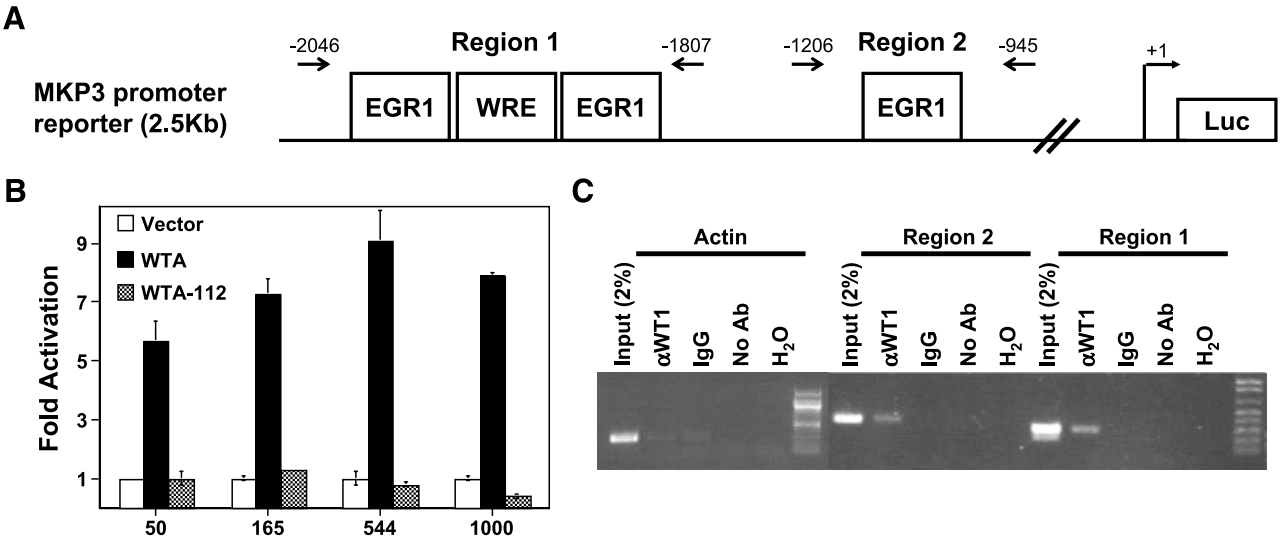

FIGURE 2. Regulation of MKP3 promoter activity by WT1 in kidney cell lines. **A.** Schematic diagram of the MKP3 promoter fragment highlighting the location of putative WT1-binding sites and PCR primers. **B.** Luciferase assays of 293T cells transfected with the MKP3 promoter and increasing concentrations of a vector expressing wild-type or mutant WT1. Data are representative of three experiments, each done in triplicate and normalized to protein concentrations. **C.** Chromatin immunoprecipitation from the Wilms' tumor cell line, CCG99-11, using a WT1 antibody, IgG, or no antibody (*Ab*). PCR primers detect MKP3 promoter regions. Actin primers were used as control.

expressing endogenous WT1, confirmed that WT1 binds to regions 1 and 2 within the MKP3 promoter (Fig. 2C). Enrichment was stronger for region 1, which contains three canonical WT1-binding sites, including the more potent WTE site, than for region 2, which covers only one EGR1-type site.

Expression of WT1 Attenuates the Activation of ERK1/2

Because WT1 transactivated MKP3 and Spry1, both inhibitors of the Ras/MAPK pathway (19), we interrogated the effect of WT1 on the phosphorylation state of ERK1/2 in the Saos-2 cells by Western blot and detected a decrease in phosphorylated ERK1/2, but not total ERK1/2, levels on induction of WT1 (data not shown). This phenomenon, coupled with the fact that growth factor–induced MKP3 expression antagonizes MAPK signaling in the developing vertebrate limb (34), prompted us to study the effect of MKP3 induction by WT1 on the phosphorylation state of ERK1/2 in kidney-related cell lines. CCG99-11 Wilms' tumor–derived cells and HEK293 kidney cells were engineered to overexpress WT1A in an inducible manner. Induction of WT1 in the CCG99-11 cells significantly attenuated the ability of EGF to induce phosphorylation of ERK1/2 (Fig. 3A). Of note, a previous study showed that WT1 repressed the EGFR expression at the transcriptional level in U2OS and Saos-2 cells resulting in apoptosis (16). However, our expression array profiles from CCG5.1 cells showed that the expression levels of all 15 EGFR probe sets on HG U133Plus2 were not changed after WT1 induction (data not shown), suggesting that the decrease or lack of EGF response was not due to a decrease in EGFR. The HEK293-inducible cell line clones exhibited a high level of basal phosphorylated ERK1/2 without EGF stimulation (Fig. 3B, *lanes 1* and *5*). Nonetheless, this basal phosphorylated ERK1/2 was depressed on induction of WT1 (Fig. 3B, *lanes 2* and *6* and C, *lane 2*).

To explore the role of MKP3 in the pathway between WT1 and ERK1/2, a catalytically inactive dominant-negative form of MKP3 (C294S), which binds to phosphorylated ERK1/2 but lacks phosphatase activity (34, 35), was transfected into the HEK293-inducible clones. Under this condition, the level of basal phosphorylated ERK1/2 was dramatically increased (Fig. 3B, *lanes 3* and *7*) and WT1 was less effective at blocking ERK1/2 phosphorylation [compare *lanes 1* and *2* (2.5:1) with *lanes 3* and *4* (1.3:1)], consistent with the notion that MKP3 mediates, at least in part, the effect of WT1 on phosphorylated ERK1/2. Short hairpin RNA (shRNA)-mediated knockdown of MKP3 (Figs. 3C, *lanes 3* and *4* and 4) resulted in a large increase in basal phosphorylated ERK1/2 levels (Fig. 3C, *lane 3*). Furthermore, when MKP3 was depleted, the inhibitory effect of WT1 on phosphorylated ERK1/2 levels was attenuated (Fig. 3C, compare *lanes 1* and *2* with *lanes 3* and *4*).

Tumor Suppressor Activity of WT1 Uses MKP3

We previously showed that WT1 inhibited the oncogenicity of activated Ras in an NIH3T3 focus-forming assay (36). We reconfirmed that WT1 blocked the tumor-promoting activity of Ras and had no tumor-promoting activity itself (Fig. 5A). Similarly, Flag-tagged MKP3, when coexpressed with Ras, blocked Ras-mediated foci formation (Fig. 5A). The MKP3 C294S mutant could not be used in this assay because it still interferes with the translocation of active ERK1/2 to the nucleus, although it lacks phosphatase activity (34, 35). Therefore, a shRNA targeting murine MKP3 was developed that depleted endogenous MKP3 mRNA by 60% (Fig. 4). This moderate depletion did not promote foci formation and did not affect Ras-induced foci formation (Fig. 5B). However, in the presence of the MKP3 shRNA, WT1 was less effective in inhibiting focus formation by Ras.

Discussion

In this study, we identified *MKP3* as a novel target gene of WT1 that mediates WT1 inhibition of ERK1/2 activation. We found that induction of WT1 blocked activation of ERK1/2 in Saos-2 and other cell lines. This was associated with a striking elevation of MKP3 levels. We showed that overexpression of WT1 induced expression of MKP3 *in vivo* and bound to and transactivated the promoter of MKP3. Furthermore, using shRNA methodology, we showed that MKP3 is an intermediary in the pathway between WT1 and ERK phosphorylation. We have previously shown that WT1 augments levels of Spry1 that can inhibit ERK1/2 activation by growth factors as well (19). Both MKP3 and Spry1 seem to be direct targets of WT1. Whether other counterregulatory molecules are directly bound and activated by WT1 or are indirectly activated through induction of growth factors remains to be determined.

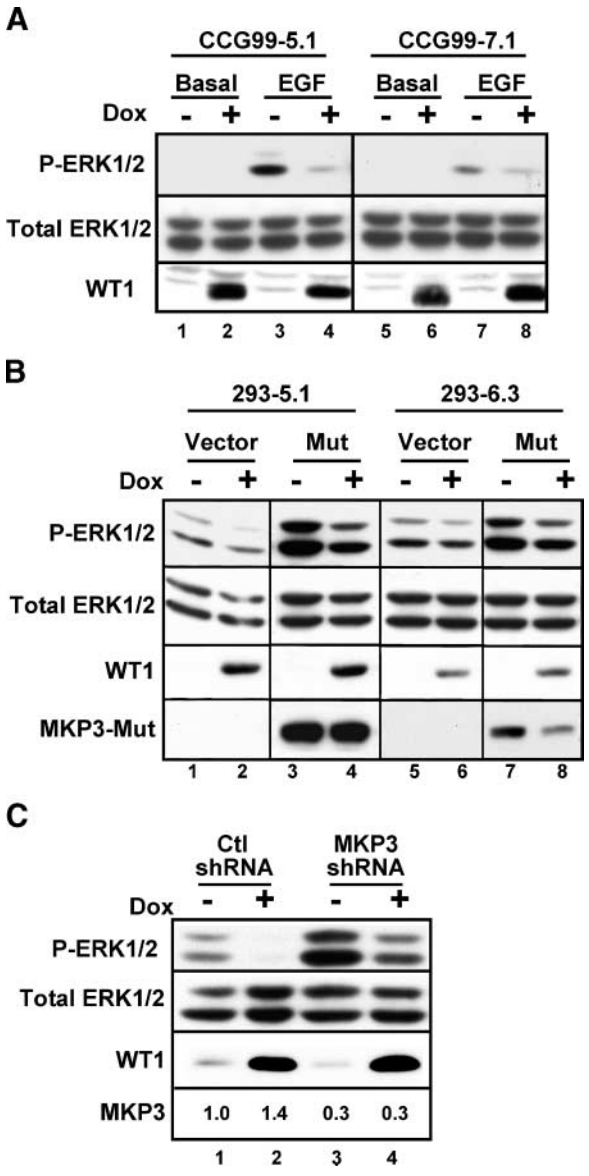

FIGURE 3. WT1 regulates the level of phosphorylated ERK1/2 (*P-ERK1/2*) through activation of MKP3. **A.** WT1-inducible CCG99-11 clones were incubated with or without doxycycline (*Dox*) in the presence or absence of EGF. HEK293 clones were transfected with either (**B**) Flag-tagged MKP3 C294S mutant or (**C**) MKP3 shRNA vectors before induction with doxycycline. Western blots were done to detect total and phosphorylated ERK1/2, WT1, and MKP3. Relative expression of endogenous MKP3 mRNA, as determined by quantitative PCR, is indicated.

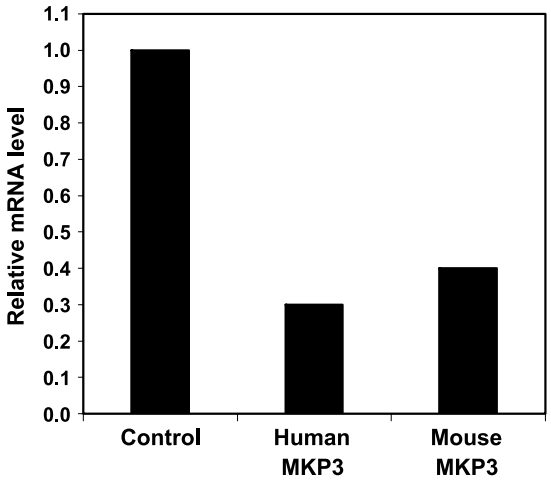

FIGURE 4. Reduction of MKP3 expression by shRNA. HEK293 and 3T3 cell lines were transfected with human and mouse MKP3-specific shRNA vectors, respectively, or with control vector and selected for 4 d. Expression level of MKP3 was determined by quantitative PCR analysis.

WT1 has been described as both a tumor suppressor and tumor promoter, depending on the cell type or the developmental stage in which WT1 is expressed (reviewed in ref. 37). For example, at murine embryonic day 9, WT1 expression is required for maintenance of the metanephric mesenchyme (reviewed in ref. 38). In the absence of WT1 expression, the metanephric mesenchyme undergoes apoptosis and the ureteric buds fail to grow. However, by day 11, if WT1 expression is knocked down by small interfering RNA, nephrogenesis is severely impaired and abnormal cell proliferation occurs (39). Overproliferation of cells that do not undergo apoptosis and persist in the form of nephrogenic rests can become the basis of a tumor. During hematopoiesis in adult tissues, overexpression of WT1 leads to growth arrest and reduced colony formation, suggesting a role as tumor suppressor (40). Furthermore, 10% to 15% of acute myelogenous leukemia patients have mutations in WT1 and overexpression of WT1 in a WT1-negative myeloblastic leukemia cells results in decreased cell growth (37). Conversely, other studies reported WT1 up-regulation in the majority of cases of acute leukemia. In model acute myelogenous leukemia systems, a knockdown of WT1 was associated with decreased growth and increased apoptosis. In addition, overexpression of WT1 in K562 erythroleukemia cells actually protected the cells against induction of apoptosis by chemotherapy agents, whereas WT1-specific small interfering RNA retarded proliferation of the cell line (41), suggesting a role as an oncogene.

These varying assignments of WT as a tumor suppressor or oncogene may be due to the variety of its interacting proteins. For example, coexpression of p53 with WT1 can block the proapoptotic activity of p53 and convert WT1 from a transactivator to a transrepressor (42, 43). Furthermore,

multiple WT1 isoforms and posttranslational modifications, such as phosphorylation and sumoylation, can regulate WT1 function. Phosphorylation of WT1 causes redistribution of the protein to the cytoplasm and blocks the ability of WT1 to bind DNA (44). Mutant WT1 molecules can dimerize with the wild-type form through an NH_2-terminal association domain and act as a dominant-negative inhibitor of WT1 function (45).

Given its role as a negative regulator of ERK1/2, *MKP3* can be considered a putative tumor suppressor gene. Marchetti and colleagues (46) found that MKP3 blocked oncogenic Ras activity *in vivo*. This group transformed a doxycycline-inducible MKP3 cell line with HA-Ras and injected it into nude mice. Mice treated with doxycycline experienced a delay in tumor emergence and reduced growth. MKP3 maps to 12q22, a locus that is either lost or hypermethylated in human pancreatic cancer (47, 48). Furthermore, *in situ* carcinomas express elevated levels of MKP3, whereas MKP3 is down-regulated in invasive carcinomas. MKP3 is a target of palytoxin, a skin tumor-promoting agent (49), which up-regulates the activity of ERK by inducing the loss of MKP3 protein, in the presence of oncogenic Ras, through an unknown mechanism. Collectively, these data suggest that induction of MKP3 may represent a therapeutic goal in cancer.

Materials and Methods

Plasmids

To generate the MKP3-luc reporter, a 2-kb section of the human MKP3 promoter (Ensemble Gene ID ENSG00000139318) from −2,140 to +20, relative to the start of transcription, was PCR amplified from human genomic DNA with gene-specific primers (5′-ATAGGTACCCGAACACGCTCCTC-CAGG-3′ and 5′ TTTAAGCTTAATCCCTCCCTCCAAGGC), introducing *Kpn*I and *Hin*dIII sites, and cloned into pGL2-Basic (Promega). Flag-tagged wild-type and mutant hMKP3 were subcloned from GST-hMKP3 and GST-hMKP3 (C294S; gift of Ming-Ming Zhou, Mount Sinai School of Medicine, New York, NY) and ligated to pCMV-Flag (Invitrogen). pBIG2i-WT1A, a doxycycline-inducible vector, was previously described (33). AU5-RasR12 was provided by Andrew Chen (Mount Sinai School of Medicine) and WT1A and WT1A-112 expression vectors were previously described (4).

Cell Culture

WT1A doxycycline-inducible cell lines were established in HEK293 and CCG99-11 cells by transfection with pBIG2i-WT1A using Lipofectamine Plus (33). Transformants were selected in 100 or 400 μg/mL of hygromycin B and expanded and WT1 expression was induced in 2 or 1 μg/mL of doxycycline, respectively. Saos-WT1–inducible clones (11) were maintained in DMEM containing 10% fetal bovine serum, 0.5 mg/mL G418, and 1 μg/mL tetracycline and induced by tetracycline withdrawal. NIH3T3 cells were maintained in DMEM containing 10% newborn calf serum, and HEK293 and 293T cells were maintained in DMEM containing 10% fetal bovine serum.

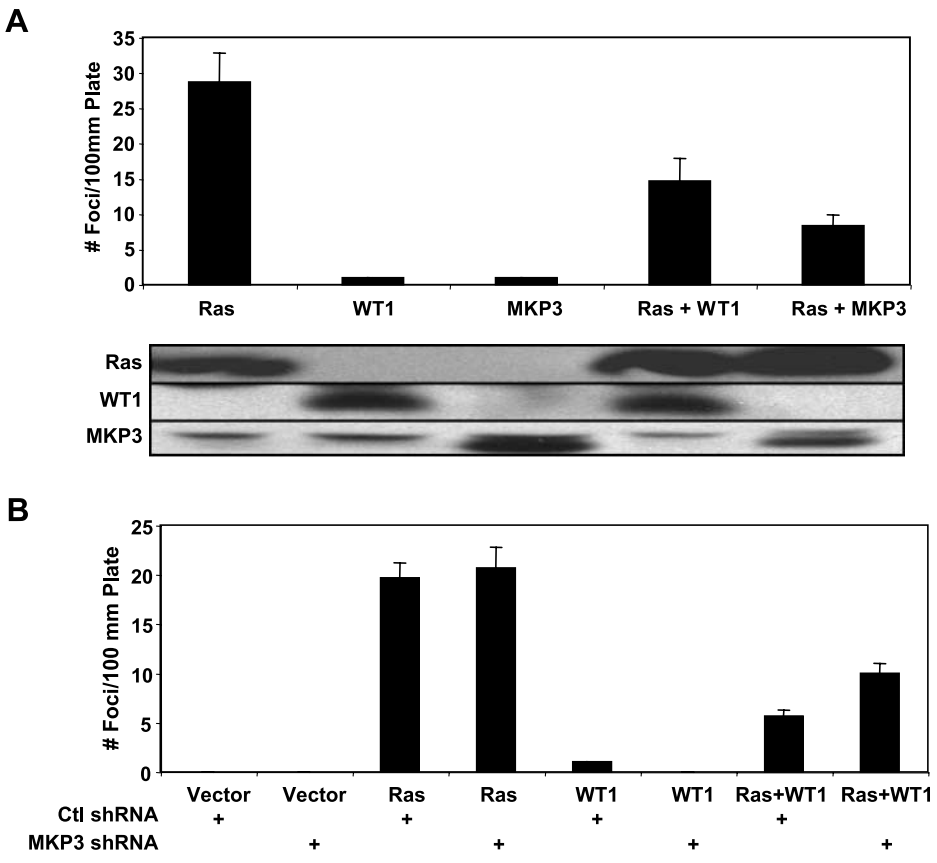

FIGURE 5. Suppression of Ras-induced foci formation by WT1 is mediated by MKP3. **A.** NIH3T3 cells were transfected with AU5-Ras in the presence or absence of WT1 or Flag-tagged MKP3 and allowed to grow for 10 d. Foci were counted and plotted as mean number of foci per 10-cm plate. The relative expression of each protein, as determined by Western blot, is shown. **B.** Knockdown of endogenous MKP3 expression by shRNA partially blocked the WT1-mediated suppression of foci formation. Experiments were carried out independently thrice in triplicate.

Quantitative Reverse Transcription-PCR Analysis

RNA was extracted using RNeasy (Qiagen) and reverse transcription-PCR was done using iScript II cDNA Synthesis kit (Bio-Rad) and QuantiTect SYBR Green PCR kit (Qiagen). Synthesis of PCR products was monitored by the DNA Engine Opticon System (MJ Research) and normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) amplification. The primers were the following: hGAPDH (5′-CCAAAATCAAGTGGGGCGATG-3′ and 5′-AAAGGTGGAGGAGTGGGTGTCG-3′), hDUSP6 (5′-CAACAGGGTTCCA-GCACAGCAG-3′ and 5′-GCCAGACACATTCCAGCAAGGAG-3′), mDUSP6 (5′-TCGGGCTGCTGCTCAAGAAAC-3′ and 5′-CGGTCAAGGTCAGACTCAATGTCC-3′), hETV1 (5′-TCCCTCCATCGCAGTCCATACC-3′ and 5′-TCCTTCCCTTGGCATCGTCG-3′), hETV4 (5′-TCAAACAGGAACAGACGGACTTCG-3′ and 5′-TCAGGGACAACGCAGACATCATC-3′), and hETV5 (5′-CCTGATGATGAACAGTTTGTCCCAG-3′ and 5′-CCATAGTTAGCACCAAGAGCCTGC-3′).

Immunoblotting

Cell lysates were prepared in 1% NP40 lysis buffer [150 mmol/L NaCl, 50 mmol/L Tris (pH 6.9), 1% NP40, 1 mmol/L phenylmethylsulfonyl fluoride, 10 mmol/L NaF, 15 mmol/L EDTA, 1 mmol/L Na_3VO_4, 20 mmol/L $Na_4P_2O_7$, protease inhibitor cocktail (Roche Diagnostics)]. Immunoblotting was done with the following antibodies: WT1 (C-19; Santa Cruz Biotechnology), GAPDH (Chemicon International), phosphorylated ERK1/2 (E-4; Santa Cruz Biotechnology), ERK1/2 (Upstate), AU5 (Covance), and Flag (F3165; Sigma). For induction of phosphorylated ERK, CCG-5.1 and 7.1 were treated with EGF (40 ng/mL) for 10 min at 37°C before lysis.

Luciferase Assay

MKP3-luc (100 ng), a control *Renilla* reporter (5 ng), and increasing concentrations of WT1A expression vectors (1 μg) were transfected into 293T cells using SuperFect (Qiagen). Cells were harvested after 48 h and assayed using the Dual-Luciferase Reporter Assay System (Promega). Results were normalized to total protein concentration determined by Bradford assay.

Chromatin Immunoprecipitation

Chromatin immunoprecipitation was done (33) with anti-WT1 (C-19), a matched IgG isotype, or no primary antibody at a concentration of 3 μg per 1.5×10^7 cells. The PCR contained 2 μL of either immunoprecipitated sample or 2% of input DNA, 0.4 μmol/L of each primer, and 12.5 μL of 2× SYBR Green Mix (Qiagen) in a 25 μL reaction. After 40 cycles of amplification, the PCR product was resolved on a 1% agarose gel. The primer sequences for MKP3 promoter regions are as follows: MKP3-1, 5′-CATTTCCCAAACCAGGGAAGAG-3′ (forward) and 5′-AAGAGGCCGCGCTTTGTCCCA-3′ (reverse); MKP3-2, 5′-GCCAACTGTAACCAATCGTCGA-3′ (forward) and 5′-GTTCGGGGGAATCTATATCTCTC-3′ (reverse); and β-actin, 5′-CCTCTTCCTCAATCTCGCTCT-3′ (forward) and 5′-CTCGAGCCATAAAAGGCAACT-3′ (reverse).

RNA Interference

shRNAs targeting MKP3 (mouse: 5′-AGCTCAACCTGTCCATGAA-3′; human: 5′-GCTCAATCTGTCGATGAAC-3′) were ligated to the pSiren-RetroQ (puromycin selection) vector (Clontech). A negative control shRNA was provided by Clontech.

Foci Formation Assay

NIH3T3 cells were transfected with the indicated combinations of AU5-RasR12, WT1, Flag-MKP3, control shRNA, and mMKP3 shRNA using Lipofectamine Plus (Invitrogen). At 24 h after transfection, plates were split 1:3 and cultured for 10 d until foci formed. Cells were fixed (10% methanol, 10% acetic acid) and stained with 0.4% crystal violet in 10% ethanol, and foci were counted blindly. For Western blotting, cells were harvested at 48 h after transfection and processed as above. In shRNA experiments, cells were selected in puromycin (1.0 μg/mL) for 4 d following transfection. Surviving cells were grown for an additional 10 d before staining.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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