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Molecular Cancer Research

FOXD3 Regulates Migration Properties and Rnd3 Expression in Melanoma Cells

Pragati Katiyar and Andrew E. Aplin

Abstract

Forkhead transcription factor, Foxd3, plays a critical role during development by controlling the lineage specification of neural crest cells. Notably, Foxd3 is highly expressed during the wave of neural crest cell migration that forms peripheral neurons and glial cells but is downregulated prior to migration of cells that give rise to the melanocytic lineage. Melanoma is the deadliest form of skin cancer and is derived from melanocytes. Recently, we showed that FOXD3 expression is elevated following the targeted inhibition of the B-RAF–MEK (MAP/ERK kinase)–ERK (extracellular signal-regulated kinase)1/2 pathway in mutant B-RAF melanoma cells. Because melanoma cells are highly migratory and invasive in a B-RAF–dependent manner, we explored the role of FOXD3 in these processes. In this study, we show that ectopic FOXD3 expression inhibits the migration, invasion, and spheroid outgrowth of mutant B-RAF melanoma cells. Upregulation of FOXD3 expression following inhibition of B-RAF and MEK correlates with the downregulation of Rnd3, a Rho GTPase and inhibitor of RhoA–ROCK signaling. Indeed, expression of FOXD3 alone was sufficient to downregulate Rnd3 expression at the mRNA and protein levels. Mechanistically, FOXD3 was found to be recruited to the *Rnd3* promoter. Inhibition of ROCK partially restored migration in FOXD3-expressing cells. These data show that FOXD3 expression downregulates migration and invasion in melanoma cells and Rnd3, a target known to be involved in these properties. *Mol Cancer Res; 9(5); 545–52. ©2011 AACR.*

Introduction

Transformation of melanocytes gives rise to melanoma, which is the deadliest form of skin cancer. In contrast to other cancers for which the overall incidence has decreased in recent years, melanoma has shown an alarming increase in the number of new cases. Melanocytes reside in the epidermis, the outermost layer of the skin. During transformation, cells accumulate in the epidermis and papillary dermal layer as radial growth phase tumors. Following the acquisition of invasive properties, vertical growth phase melanoma cells invade en masse down into the underlying dermis. The depth of invasion is used as a clinical correlate for a poor prognosis (1). Thus, it is critically important to understand the mechanisms that regulate the migratory and invasive properties of melanoma.

Activating mutations in the serine-threonine kinase B-RAF occur in approximately 60% of melanoma cases (2). The most frequent mutation, *B-RAF*^{V600E}, results in elevated kinase activity that is independent of upstream RAS signaling (2). In this manner, B-RAF^{V600E} stimulates extracellular signal-regulated kinase (ERK) signaling, leading to increased cell proliferation and survival (3, 4). In addition, depletion of B-RAF by RNA interference inhibits melanoma cell invasion through Matrigel (5). The molecular mechanisms underlying these growth and invasive phenotypes are yet to be clearly determined; however, they are likely to be complex because *B-RAF* mutations have also been detected in nonmalignant nevi. Notably, the levels of phospho-ERK1/2 (pERK1/2) are low in nevi, likely due to feedback inhibition (6).

Cell migration and invasion are regulated by the family of Rho proteins including RhoA, Rac1, and Cdc42 that work as molecular switches to ultimately regulate actin cytoskeletal structures (7). Rnd3, also known as RhoE/Rho8, is an additional member of the Rho family (8). Unlike other Rho family members, Rnd3 lacks GTPase activity resulting in its constitutive GTP binding. Notably, Rnd3 binds p190RhoGAP to antagonize RhoA functions and inhibit actin stress fiber formation (9). Rnd3 is highly expressed in invasive melanoma cells (10). Recently, we have shown Rnd3 is positively regulated by B-RAF^{V600E}–MEK (MAP/ERK

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Note: Throughout this article, FOXD3, Foxd3, and FoxD3 refer to the human, mouse, and all other chordate species form of this protein, respectively.

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kinase) signaling (11). Furthermore, we showed that Rnd3 regulates the actin cytoskeleton and is required for melanoma cell migration and invasion in 3-dimensional (3D) collagen (12).

Foxd3 is a member of forkhead box (Fox) transcription factor family that is characterized by the presence of a forkhead domain (13). It binds to DNA with the consensus sequence 5′-A [AT]T[AG]TTTGTTT-3′ (14). Role of Foxd3 in development is well established, as it has been shown to be expressed in embryonic stem (ES) cells in the late-stage gastrula inner cell mass (epiblast; ref. 15) and is required for extra embryonic tissue (16). Importantly, Foxd3 is essential during normal murine development by maintaining pluripotent cells in the early mouse embryo and is required to establish murine ES cell lines *in vitro* (15, 17, 18). A role for Foxd3 later in development has also been established, specifically in premigrating and migrating neural crest cells in avian embryo (19, 20). Foxd3 is an early molecular marker of neural crest cells and is responsible for the repression of melanogenesis in early migratory neural crest cells (19). Interestingly, overexpression of Foxd3 in late migrating neural crest cells that are destined for melanoblast formation results in a shift toward glial and neural cell lineages (19, 21, 22). Recently, our laboratory showed that FOXD3 is upregulated by inhibition of the B-RAF–MEK pathway in mutant B-RAF melanoma cells and that ectopic expression of Foxd3 in melanoma cells induces a G_1-S phase arrest (23). Because Foxd3 has been implicated in the migration and invasion in neural crest cells, we tested its role in the regulation of migration and invasion in mutant B-RAF melanoma cells.

Materials and Methods

Cell culture

Human mutant B-RAF WM793 and wild-type B-RAF WM3211 melanoma cell lines were kindly donated by Dr. Meenard Herlyn (Wistar Institute, Philadelphia, PA) and were cultured in MCDB 153 medium containing 20% Leibovitz L-15 medium, 2% FBS, 0.2% sodium bicarbonate, and 5 μg/mL insulin. A375 cells were purchased from American Type Culture Collection and were cultured in DMEM with 10% FBS. The generation of WM793TR and A375TR cell lines that inducibly express β-galactosidase (LacZ), mFoxd3, and hFOXD3 has been previously described (23). Transgene expression was induced by the addition of 100 ng/mL doxycycline to the medium.

Antibodies and inhibitors

Primary antibodies used were as follows: ERK1/2 (K-23; Santa Cruz Biotechnology, Inc.); pERK1/2 (E10; Cell Signaling Technology); FOXD3 (polyclonal 6317; BioLegend), V5 Tag (46-0705; Invitrogen); trimethyl histone H3 [(Lys4); H3K4, #9751; Cell Signaling Technology]; RNA polymerase II (Pol II) CTD repeat YSPTSPS [(phospho-S2); ab5095; Abcam]; and phospho-myosin light chain (pMLC; #3675; Cell Signaling Technology). PLX4720 was kindly donated by Plexxikon Inc. AZD6244 was purchased from Selleck. U0126 was obtained from Cell Signaling Technology. Y27632 was purchased from Calbiochem.

Western blotting

Cells were lysed and lysates analyzed by Western blotting, as previously described (24). Chemiluminescence was detected on a Versadoc Multi-Imager and quantitated using Quantity One software (Bio-Rad).

Migration and invasion assays

Migration and invasion were assayed by seeding (2.5–3) $\times 10^4$ cells on top of Boyden chamber insert or Matrigel-coated cell culture inserts (BD Biosciences), respectively. Serum-free medium was added to the top chamber, and serum-containing medium to the bottom chamber. Cells were allowed to migrate at 37°C for 6 hours before fixation. Membranes were stained and cells were counted from 5 different fields. The average number of migrating cells was taken from 3 independent experiments.

Spheroid outgrowth assay

Three independent spheroid assays were carried out, as previously described (25). Briefly, 5×10^4 cells were seeded in suspension in full serum medium on top of a 2% bactoagar layer and spheroids were allowed to form for 72 hours at 37°C. Collected spheroids were embedded in 3D collagen and incubated at 37°C for 2 hours to solidify, and medium was added on top of the collagen. Photographs of spheroids were taken 24 hours after collagen embedding by using a Nikon Eclipse Tsi inverted microscope with 10× magnification. Next, 100 ng/mL doxycycline was added for a further 48 hours. At least 10 spheroids were analyzed for each condition. At the end of 48 hours, live/dead staining of spheroids was carried out using a viability/toxicity kit (Invitrogen) as per manufacturer's instructions.

Quantitative reverse transcription PCR

Total RNA was extracted by using PerfectPure RNA cultured cell kit (5 Prime). cDNA was prepared by using iScript cDNA synthesis kit (Bio-Rad). Quantitative PCR was carried out with iQ SYBR Green supermix (Bio-Rad), using 400 nmol/L primers, and 0.1 μg cDNA. The primers used to detect Rnd3 were as follows: forward 5′-CGTTAAGCGGAACAAATCACAG-3′ and reverse 5′-CGTAAGTCCGTAGCAACTGC-3′. For actin, the primers were as follows: forward 5′-AGTGTGGTCCTGC-GACTTCTAAG-3′ and reverse 5′-CCTGGGCTTGAGA GGTAGAGTGT-3′. Reaction conditions were denaturation at 94°C for 30 seconds, annealing at 50°C for 30 seconds, and elongation at 72°C for 30 seconds (50 cycles in total). PCR was done on an iCycler with MyiQ version 1.0 software (Bio-Rad). Relative mRNA levels were calculated using the comparative C_t method (26).

Chromatin immunoprecipitation

A375TR/mFoxd3 cells were treated with 100 ng/mL doxycycline for 48 hours. Cross-linking of the DNA–protein

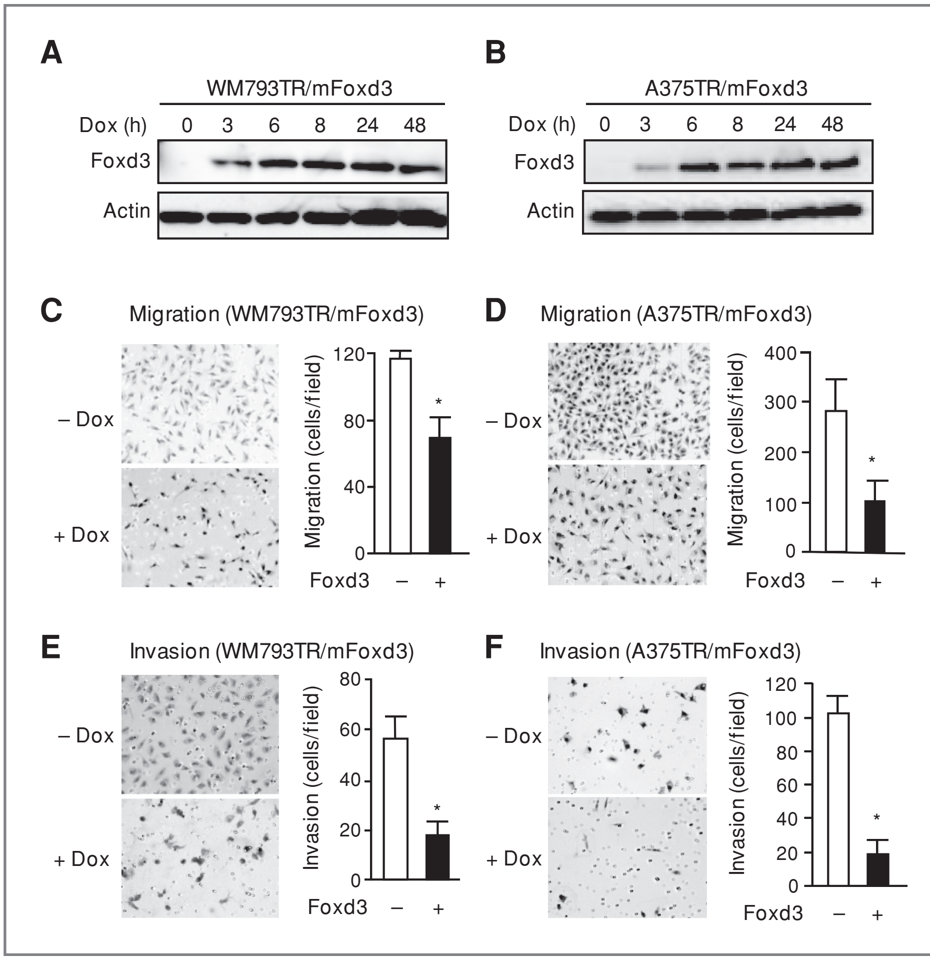

Figure 1. Foxd3 induction reduces migration and invasion in mutant B-RAF melanoma cells. A, inducible WM793TR/mFoxd3 cells were treated with 100 ng/mL doxycycline (Dox) for the indicated time points. Cell lysates were analyzed for Foxd3 expression by Western blotting. Actin serves as a loading control. B, as in A, except A375TR/mFoxd3 cells were used. C, WM793TR/mFoxd3 cells were allowed to migrate through uncoated Boyden chambers for 6 hours. Representative pictures for the underside of the filters are shown. Quantitation of cells which have migrated through the Boyden chamber is the mean and SD from 3 independent experiments. D, as in C, except that A375TR/mFoxd3 cells were used. E, WM793TR/mFoxd3 cells were allowed to invade overnight through Matrigel-coated chambers. F, in for E, except that A375TR/mFoxd3 cells were used. *, $P < 0.005$

complexes was carried out by addition of 37% formaldehyde and incubation at 37°C for 10 minutes. Reactions were stopped by the addition of 1 mL of 1.25 mol/L glycine solution for 5 minutes. Cells were then washed in PBS containing protease inhibitors (protease inhibitor cocktail set III; Calbiochem) and lysed by the addition of SDS lysis buffer (1% SDS, 10 mmol/L EDTA, and 50 mmol/L Tris-HCl, pH 8.1). The lysates were sonicated on ice for 4 cycles, with each cycle consisting of 10 pulses of 1 second each at 30% amplitude, followed by a 30-second interval between each cycle by using a Sonic Dismembrator 500 (Fisher Scientific). After sonication, the lysates were centrifuged and the supernatant was diluted 10-fold with chromatin immunoprecipitation (ChIP) dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mmol/L EDTA, 16.7 mmol/L Tris-HCl, pH 8.1, 167 mmol/L NaCl), and 1% of the diluted lysate was used as input for DNA quantitation. Preclearing was done overnight at 4°C by incubating lysate with protein A/G agarose beads (Santa Cruz Biotechnology). Primary antibodies for ChIP were conjugated with protein G magnetic beads (Dynabeads Protein G; Invitrogen) at 4°C for 1 hour and then added to precleared lysates for 4 hours at 4°C. Following washing with low salt buffer, high salt buffer, LiCl, and TE buffer, immunoprecipitated protein–DNA complexes were eluted from the beads and subjected to reverse cross-linking at 65°C. DNA was purified using a ChIP DNA Clean and concentrator Kit (Zymo Research Corporation Orange). The purified DNA was then subjected to PCR amplification of a sequence spanning the Foxd3 consensus sequence in the *Rnd3* gene promoter. The primers used were as follows: forward 5′-GCTT-CCTATGTTTTATCACTG-3′ and reverse 5′-CTGA-TTTGAACTACAATCCC-3′. Reaction conditions were denaturation at 94°C for 30 seconds, annealing at 56°C for 30 seconds, and elongation at 72°C for 30 seconds (45 cycles in total). PCR products were visualized on 2% agarose gel.

Results

FOXD3 regulation of migration and invasion

Because of the role of Foxd3 during development, we investigated its role in the migration and invasion of melanoma cells. We focused on mutant B-RAF–harboring melanoma cells because FOXD3 is upregulated following B-RAF–MEK inhibition selectively in this genotype of melanoma cells (23). Mutant B-RAF–harboring WM793 and A375 cells were treated with doxycycline for up to 48

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hours to induce Foxd3. Induction of Foxd3 was detected within 3 hours of doxycycline treatment and remained consistent throughout the time course of the experiment (Fig. 1 A and B). After 48 hours, WM793 and A375 cells were seeded on uncoated Boyden chambers for migration assays and on Matrigel-coated Boyden chambers for invasion assays. Quantitative analysis of 3 independent experiments indicated that Foxd3 expression caused a significant decrease ($P < 0.005$) in both migration (Fig. 1 C and D) and invasion in WM793 and A375 cells (Fig. 1 E and F).

Foxd3 reduces the spheroid outgrowth in 3D collagen in mutant melanoma cells

Type I collagen is the major extracellular matrix (ECM) component of the dermis; therefore, we measured the effect of Foxd3 expression on melanoma cell spheroid outgrowth in 3D collagen. WM793 cells were grown on top of bactoagar in suspension and the resulting spheroids were embedded in 3D type I collagen. Spheroids composed of control WM793TR/LacZ cells showed a characteristic increase in size and outgrowth into the surrounding collagen matrix in both minus and plus doxycycline treatment conditions (Fig. 2A, left 2 panels). Spheroid outgrowth in WM793TR/mFoxd3 cells in the absence of doxycycline

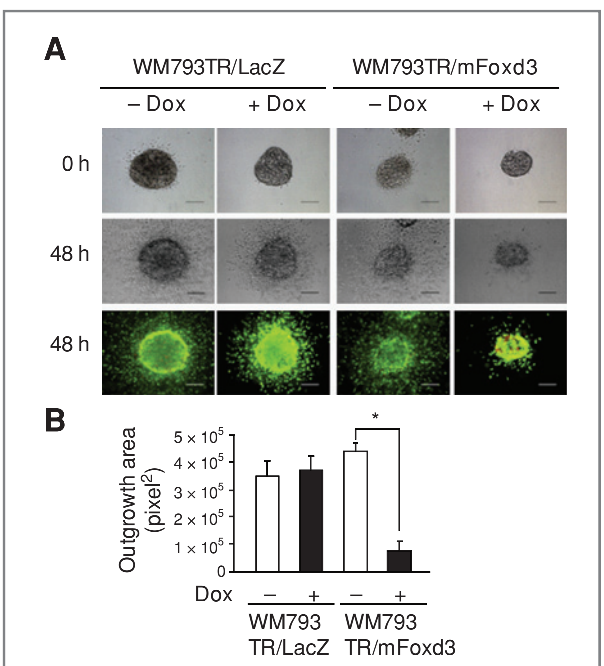

Figure 2. Foxd3 regulates melanoma spheroid outgrowth in 3D collagen. A, spheroids from WM793TR/LacZ and WM793TR/mFoxd3 cells were embedded in collagen and treated ± doxycycline (Dox) for 48 hours to induce Foxd3. After 48 hours, live (green)/dead (red) staining of cells was done. A representative image for each condition from 1 of 3 independent experiments is shown. Scale bars, 200 μm. B, quantitation of spheroid outgrowth. Spheroid outgrowth was subtracted from the spheroid core area and normalized to $t = 0$. Three independent experiments were conducted and a minimum of 10 spheroids were quantitated for each condition per experiment. *, $P < 0.005$.

was comparable with WM793TR/LacZ cells. In contrast, Foxd3 induction in WM793TR/mFoxd3 spheroid inhibited cell outgrowth compared with WM793TR/LacZ or noninduced WM793TR/mFoxd3 cells (Fig. 2A, right 2 panels). The core area and the outgrowth of spheroids were measured at 0 hour and after 48 hours of doxycycline treatment. Core area is the circumference of the solid mass of the spheroid. For outgrowth measurements, the outer edges of the spheroid were determined. We quantitated spheroid outgrowth by subtracting the core area from the outgrowth of each spheroid. This analysis showed that FOXD3 expression resulted in a statistically significant decrease in spheroid outgrowth (Fig. 2B).

Mutant B-RAF inhibition by PLX4720 results in upregulation of Foxd3

We have previously shown that FOXD3 is upregulated upon B-RAF knockdown (23). Here, we used PLX4720, which selectively inhibits B-RAF–MEK–ERK1/2 signaling in mutant B-RAF^{V600E}–expressing melanoma cells (27). As expected, treatment of mutant B-RAF cell lines with PLX4720 inhibited pERK1/2 levels in WM793 and A375 cells (Fig. 3A). Concomitant with pERK1/2 inhibition was an increase in FOXD3 expression and a decrease in the expression of the Rho family member Rnd3 (Fig. 3A). In addition, treatment of mutant B-RAF cell lines (WM793 and A375) with the MEK inhibitor AZD6244 also resulted in an increase in FOXD3 levels and a decrease in Rnd3 expression (Fig. 3B). Inhibition of MEK in wild-type B-RAF WM3211 cells does not lead to upregulation of FOXD3 (23). Parallel to the lack of effect on FOXD3, Rnd3 was not downregulated by MEK inhibition in WM3211 cells (Fig. 3C). These data show that FOXD3 and Rnd3 are regulated in an opposing manner by the B-RAF signaling pathway in mutant B-RAF melanoma cells.

Foxd3 expression downregulates Rnd3

To determine whether Foxd3 can directly regulate Rnd3, we induced expression of mouse Foxd3 in mutant B-RAF WM793 cells. Induction of Foxd3 expression resulted in a significant decrease in Rnd3 expression in comparison with untreated cells (Fig. 4A). Human and mouse forms of FOXD3 are more than 88% identical; nevertheless, to confirm whether the human FOXD3 acted similarly, we used WM793TR/hFOXD3 cells. Expression of both epitope-tagged and nontagged versions of human FOXD3 efficiently downregulated Rnd3 levels (Fig. 4B). In further time course analysis, WM793TR/hFOXD3-V5 cells were treated with doxycycline for increasing time intervals (Fig. 4C). Notably, Rnd3 expression was downregulated with increased expression of FOXD3. No downregulation of Rnd3 was detected following doxycycline treatment of control WM793TR/LacZ-V5 cells. To test the effect of Foxd3 expression on Rnd3 RNA level, total RNA was isolated from WM793TR/LacZ and WM793TR/mFoxd3 cells treated with doxycycline for 48 hours and subjected to quantitative reverse transcriptase PCR analysis. Consistent with protein data, Rnd3 RNA was also found to be

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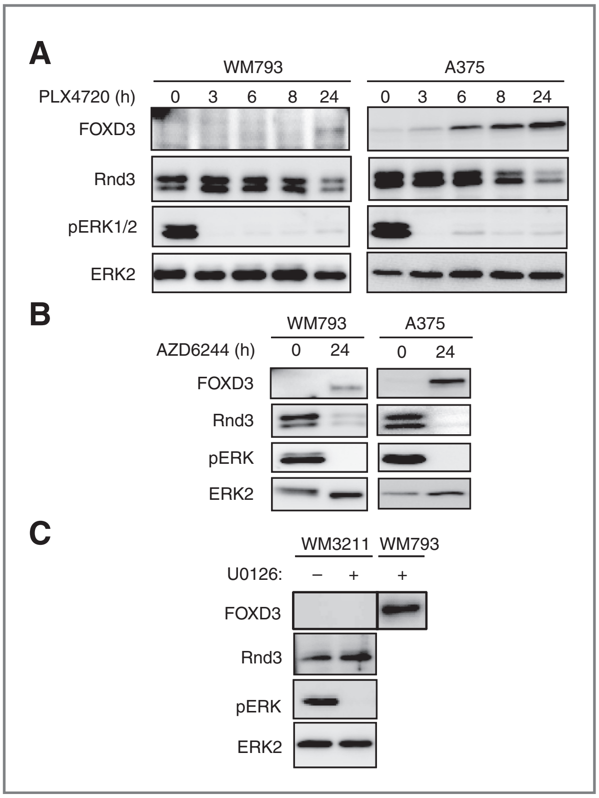

Figure 3. Disruption of mutant B-RAF signaling increases FOXD3 and decreases Rnd3 expression. A, mutant B-RAF melanoma cell lines (WM793 and A375) were treated with the B-RAF inhibitor PLX4720 (1 μmol/L) for the times indicated. Cell lysates were analyzed for the expression of FOXD3, Rnd3, pERK1/2, and total ERK2 by Western blotting. B, mutant B-RAF melanoma cell lines, WM793 and A375, were treated with the MEK inhibitor AZD6244 (3.3 μmol/L) for 24 hours. Lysates were analyzed by Western blotting, as above. C, wild-type B-RAF WM3211 cells were treated with the MEK inhibitor U0126 (10 μmol/L) for 24 hours before harvesting cells for Western blotting. Cell lysates were analyzed by Western blotting as above.

significantly decreased after Foxd3 expression (Fig. 4D). Together, these data show that Foxd3 downregulates the expression of Rnd3.

Recruitment of Foxd3 to *Rnd3* promoter

Next, we analyzed the human *Rnd3* promoter and identified a consensus forkhead transcription factor-binding site (−1,196 to −1,073). ChIP assays were carried out with primers flanking this forkhead consensus sequence to test whether FOXD3 directly binds to the *Rnd3* promoter. A schematic representation indicating the location of PCR primers flanking the FOXD3 consensus sequence in the *Rnd3* promoter is shown in Figure 5A. A375TR/mFoxd3-V5 cells were used for these experiments because we obtained consistent shearing of 500 to 1,000 bp in this cell type. The presence of an amplified band in ChIPs from A375TR/mFoxd3-V5 cells treated with doxycycline and the absence of this band from the minus doxycycline condition ChIPs indicated recruitment of Foxd3 to *Rnd3* promoter (Fig 5B). In addition, recruitment of phosphorylated RNA Pol II and methylated histone 3 (H3K4) to the *Rnd3* promoter was abrogated in cells expressing Foxd3. The absence of an amplified band in the no antibody control using normal IgG antibody indicated the specificity of ChIP (Fig 5B). Together, these data show that FOXD3 can be recruited to the human *Rnd3* promoter.

Inhibition of ROCK reverses FOXD3 inhibition of migration

Because FOXD3 reduced expression of Rnd3 and Rnd3 is an inhibitor of RhoA–ROCK signaling (28, 29), we investigated the extent to which Foxd3 affects RhoA–ROCK signaling by analysis of MLC phosphorylation. Induction of Foxd3 in WM793TR/mFoxd3 cells increased the phosphorylation of MLC (Fig. 6A). Increased MLC phosphorylation has been associated with increased actin

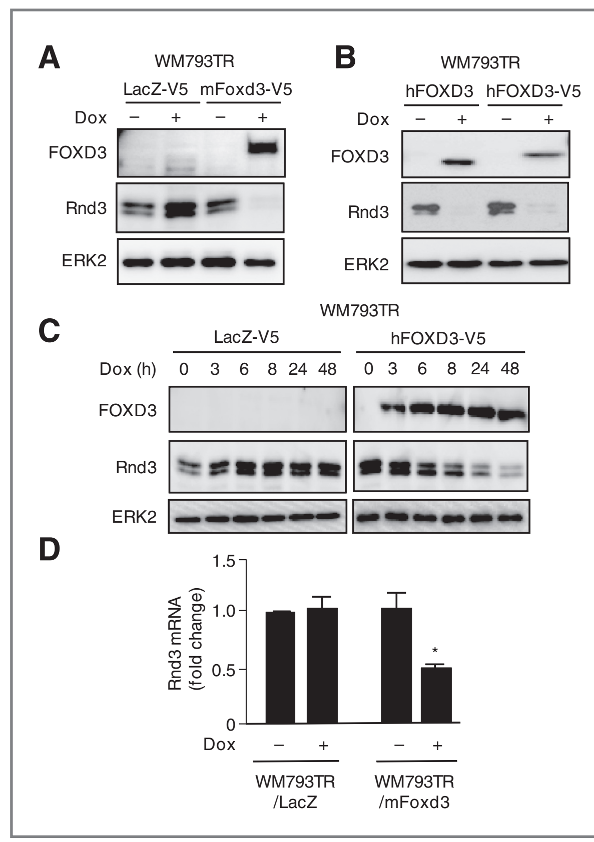

Figure 4. Foxd3 induction results in a decrease in Rnd3 expression. A, WM793TR/LacZ-V5 and WM793TR/mFoxd3 cells were treated with ± doxycycline (Dox) for 48 hours. Cell lysates were analyzed for the expression of FOXD3 and Rnd3 by Western blotting. ERK2 serves as a loading control. B, WM793TR/hFOXD3 and WM793TR/hFOXD3-V5 cells were treated with doxycycline to induce FOXD3 expression for 48 hours. Cell lysates were analyzed as in A. C, WM793TR/LacZ-V5 and WM793TR/hFOXD3-V5 cells were treated with doxycycline for indicated times. Lysates were analyzed by Western blotting as above. D, WM793TR/LacZ or WM793TR/mFOXD3 cells were treated with doxycycline for 48 hours and total RNA was extracted. cDNA was prepared from RNA and subjected to qPCR analysis for Rnd3 and actin mRNA levels. Graphs of Rnd3 mRNA/actin mRNA ratios normalized to the noninduced WM793TR/LacZ cells. Mean and SDs are from 3 independent experiments. *, $P < 0.05$.

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stress fiber formation, but FOXD3 expression did not promote actin stress fiber formation (data not shown), illustrating that FOXD3 likely has additional targets that influence the actin cytoskeleton. As expected, treatment of Foxd3-expressing WM793 cells with the ROCK inhibitor Y27632 for 48 hours reduced the Foxd3-induced increase in phosphorylation of MLC. Furthermore, the FOXD3-mediated inhibition of migration in mutant B-RAF melanoma cells was partially reversed by treatment with Y27632 (Fig. 6B). These data indicate that some of the effects of FOXD3 on migration are mediated through regulation of the RhoA–ROCK signaling pathway.

Discussion

FOXD3 is a forkhead transcription factor with well-established roles in stem cell biology and lineage specification from the neural crest. In this study, we show a role for FOXD3 in the migration and invasion of mutant B-RAF melanoma cells. Using inducible systems, we show that ectopic expression of FOXD3 inhibits melanoma cell migration, invasion through a basement membrane-like ECM, and spheroid outgrowth in 3D type I collagen. We have previously published that FOXD3 expression inhibits cell proliferation of melanoma cells through upregulation of the cyclin-dependent kinase inhibitor p21^{Cip1} (23). The effects of FOXD3 on migration and invasion seem to be independent of the cell cycle phenotype because

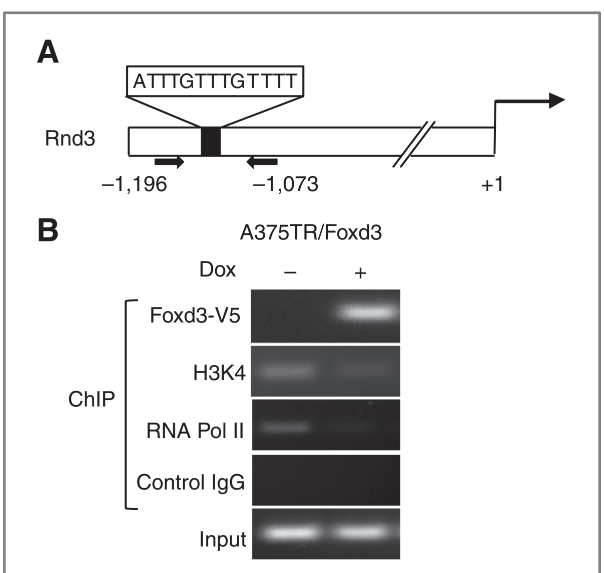

Figure 5. Recruitment of Foxd3 to *Rnd3* promoter. A, schematic representation for the location of Foxd3 consensus binding sequence in human *Rnd3* promoter. Arrows indicates primer location flanking Foxd3 consensus site. B, mutant-B-RAF melanoma A375TR/mFoxd3 cells were treated ± doxycycline for 48 hours before processing for ChIP with antibodies to V5 (to immunoprecipitate Foxd3-V5), methylated H3K4, and phosphorylated RNA Pol II. After decross-linking, PCR was carried out to test the recruitment of the *Rnd3* promoter. Input levels indicate equivalent levels of *Rnd3* promoter between the ± doxycycline conditions for A375/mFoxd3 cells.

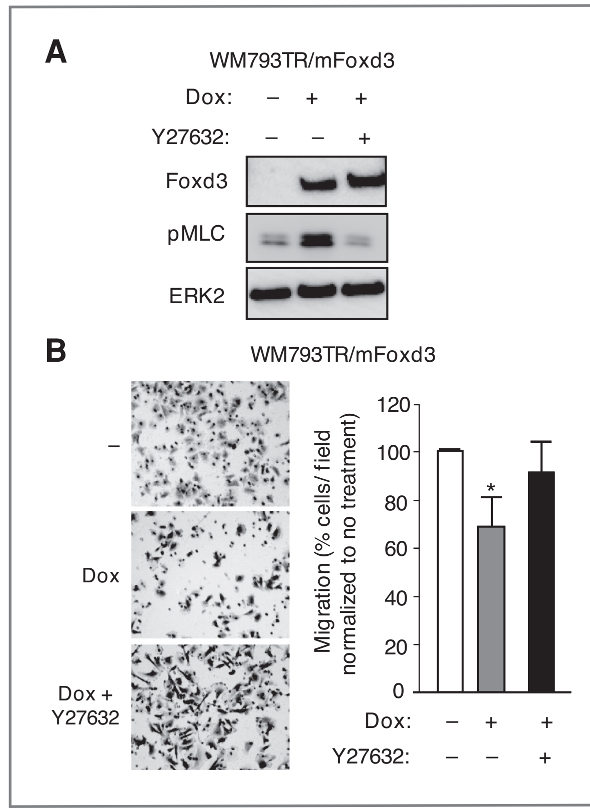

Figure 6. ROCK inhibition reverses Foxd3-mediated inhibition of migration. A, WM793TR/mFoxd3 in serum-free medium were treated ± doxycycline (Dox) and ± Y27632 (10 μmol/L) for 48 hours. Cell lysates were analyzed for the expression of Foxd3, pMLC, and ERK2. B, WM793TR/mFoxd3 were treated ± doxycycline and Y27632 for 24 hours and then drugs replenished in serum-free medium for a further 24 hours before seeding cells onto Transwell inserts. Cell migration was allowed to proceed for 6 hours at 37°C. Migrated cells were stained with crystal violet and counted under the microscope. Three independent experiments were carried out. The mean and SD value is graphed. The difference between noninduced and induced samples is statistically different (*, $P < 0.05$); however, the difference between noninduced and induced samples treated with Y27632 is not statistically different.

cell-cycle inhibition was only evident 3 to 5 days following expression, whereas effects on migration and invasion were observed within 48 hours. In addition, we have previously shown that FOXD3 is upregulated following inhibition of mutant B-RAF–MEK signaling in melanoma cells (23). We further support these data using the RAF inhibitor PLX4720, which selectively inhibits MEK–ERK1/2 signaling in mutant B-RAF cells (27). PLX4720 treatment, and subsequent inhibition of the ERK1/2 pathway, led to enhanced expression of FOXD3. B-RAF has been shown to play a role in invasion (5). B-RAF regulation of the matrix metalloproteinase MMP1 is thought to contribute to invasion through collagen (30, 31). In addition, multiple regulators of migratory and invasive properties have been identified as downstream targets of B-RAF signaling in mutant B-RAF melanoma cells. In addition to Rnd3, microarray studies have identified that B-RAF targets include IL-8, CXCL1, EphA4, PDE5A, and transcription

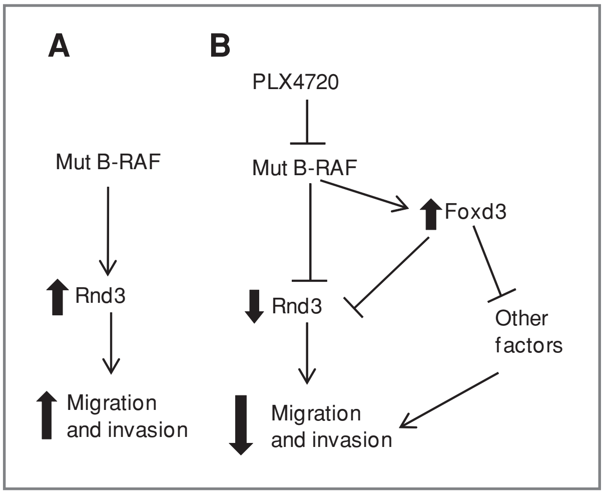

Figure 7. Schematic representation of mechanism of Foxd3-mediated inhibition of migration and invasion by inhibiting various targets involved in migration and invasion. A, mutation in B-RAF results in increased Rnd3 expression and associated with increased migration and invasion, B, B-RAF inhibitor PLX4720 inhibits B-RAF resulting in increased FOXD3 expression and decreased Rnd3 expression resulting in reduced migration and invasion.

factor FosL1/Fra1, which contribute to cell polarization, motility, and invasiveness (32–34).

In our study, we also explored the possible molecular mechanism involved in the Foxd3-mediated inhibition of migration/invasion in melanoma cells. Upregulation of FOXD3 following inhibition of B-RAF or MEK correlated with a decrease in Rnd3 expression. Rnd3 is a constitutively active, GTP-loaded Rho family member (35) that disrupts actin stress fibers and increases cellular migration (29, 36). Consistent with these findings in fibroblasts, we reported that Rnd3 knockdown in mutant B-RAF melanoma cells enhanced actin stress fiber formation and inhibited migration and invasion (12). Our data show that ectopic Foxd3 expression suppressed Rnd3 levels at both the protein and mRNA levels. In addition, Foxd3 can directly bind to the *Rnd3* proximal promoter and, in doing so, it inhibits proper assembly of transcription machinery by interfering with the recruitment of factors such as phosphorylated RNA Pol II and methylated H3K4. FOXD3 has been proposed to act as both an activator (18) and a repressor (37, 38) of transcription. Our data point to the latter role in terms of regulating Rnd3 expression in melanoma. Recent evidence highlights a number of possible mechanisms whereby FOXD3 can regulate target expression. FOXD3 expression is associated with maintenance of unmethylated marks in gene enhancers (39) and promoter CpG regions (40). Additional studies suggest interactions between Foxd3 and other embryonic stem cell factors. For example, Sox2 has been shown to establish active H3K4 di- and trimethylation marks in the enhancer of the pre-B cell-specific *λ5-VpreB1* locus but Foxd3 blocks intergenic transcription from this locus (38).

Although FOXD3 regulates Rnd3 expression, evidence indicates that additional targets are involved in FOXD3 regulation of migration and invasion. Of note, the effect of FOXD3 expression on the actin cytoskeleton differs from Rnd3 depletion. FOXD3 expression led to a spindle cell morphology with disrupted F-actin stress fibers (data not shown); whereas Rnd3 depletion enhances actin stress fiber formation (12). Thus, we believe that Rnd3 is required for migration and outgrowth of melanoma cells, based on Rnd3 knockdown experiments (12), but is not the only regulator. In summary, through this study we revealed that B-RAF inhibition results in upregulation of FOXD3 and it is likely that FOXD3 regulates Rnd3 and other targets that inhibit to the migration and invasion phenotypes in melanoma cells (Fig. 7).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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