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FOXD3 Modulates Migration Through Direct Transcriptional Repression of TWIST1 in melanoma

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Abstract

The neural crest is a multi-potent, highly migratory cell population that gives rise to diverse cell types, including melanocytes. Factors regulating the development of the neural crest and emigration of its cells are likely to influence melanoma metastasis. The transcription factor FOXD3 plays an essential role in pre-migratory neural crest development and has been implicated in melanoma cell dormancy and response to therapeutics. FOXD3 is down-regulated during migration of the melanocyte lineage from the neural crest, and our previous work supports a role for FOXD3 in suppressing melanoma cell migration and invasion. Alternatively, TWIST1 is known to have pro-migratory and pro-invasive roles in a number of cancers, including melanoma. Utilizing ChIP-seq analysis, TWIST1 was identified as a potential transcriptional target of FOXD3. Mechanistically, FOXD3 directly binds to regions of the *TWIST1* gene locus, leading to transcriptional repression of TWIST1 in human mutant BRAF melanoma cells. Additionally, depletion of endogenous FOXD3 promotes up-regulation of TWIST1 transcript and protein. Finally, FOXD3 expression leads to a significant decrease in cell migration that can be efficiently reversed by the overexpression of TWIST1. These findings uncover the novel interplay between FOXD3 and TWIST1, which is likely to be important in the melanoma metastatic cascade.

Keywords

Neural crest; BRAF; metastasis

Introduction

In order to metastasize, cancer cells may utilize the mechanisms underlying cell migration during development. Aberrant use of developmental migration mechanisms is likely to be particularly relevant in melanoma, an aggressive form of skin cancer that arises due to the

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malignant transformation of melanocytes (1, 2). During embryonic development, melanocyte precursors (melanoblasts) differentiate from neural crest stem cells and emigrate to the ectoderm to become a component of the skin (3). The highly migratory nature of melanoblasts likely coincides with the intractable and metastatic behavior of melanoma tumors. In addition, the regulation/deregulation of developmentally-linked proteins, including MITF and FOXD3, has been consistently found to drastically impact melanoma tumor biology and its response to therapeutics (4-6).

FOXD3 is a member of the forkhead box (FOX) family of transcription factors. The forkhead box is a helix-turn-helix DNA binding motif which interacts with a DNA consensus sequence of (A/C)AA(C/T)A or T(G/A)TT(T/G) with contributions from additional flanking nucleotides (7, 8). Based on DNA binding studies, FOXD3 binds the consensus sequence of A(A/T)T(A/G)TTTGTTT, which consists of two overlapping forkhead sites. While FOXD3 has been traditionally viewed as a transcriptional repressor, more recent studies highlight FOXD3 as promoting expression of a number of genes such as Nanog, Oct4, and ERBB3, suggesting dual roles in transcriptional regulation (4, 9, 10).

FOXD3 is linked to stem cell biology, particularly in the developing neural crest. Knockdown of FOXD3 has been shown to increase the level of melanoblasts emigrating from the neural crest, while overexpression of FOXD3 prevents the formation of melanoblasts (11). As such, FOXD3 participates in maintenance of “stemness” or pluripotency of melanocyte precursors and is down-regulated during the migration of the melanocyte lineage from the neural crest (11, 12).

The timing of expression of many FOX proteins, particularly FOXD3, is strictly controlled to regulate cellular processes including differentiation, proliferation, and migration; therefore, not surprisingly, FOX protein deregulation can contribute to tumorigenesis and cancer progression. While normal adult tissues typically lack detectable FOXD3, several cancers such as germ cell tumors, malignant peripheral nerve sheath tumors, schwannomas, neurofibromas, and chronic lymphocytic leukemia harbor FOXD3 protein expression changes (13-15). Given the role of FOXD3 in melanocyte precursors and its potential role in cancer development, our laboratory has undertaken studies examining the role of FOXD3 in melanoma. Importantly, we previously found that FOXD3 can act as a transcriptional activator or repressor in melanoma and abrogates migration, invasion, and spheroid outgrowth of melanoma cells (4, 16, 17). In addition, the manipulation of FOXD3 levels in melanoma leads to differential sensitivity of cells to RAF inhibition due to downstream target gene changes, such as enhanced ERBB3 expression (4, 5).

Despite these studies, the direct targets of FOXD3 remain poorly characterized and further analysis is particularly important in melanoma given the clinical relevance of FOXD3. In this study, we have uncovered TWIST1 as a novel direct downstream target of transcriptional repression by FOXD3 in melanoma. TWIST1 is a transcription factor linked to the migration of neural crest-derived cells and, in certain cell contexts, to an epithelial-to-mesenchymal transition (EMT). TWIST1 is also implicated in increased migration, invasion, metastasis, and poor prognosis in several cancers including melanoma (18-21). Importantly,

we find that suppression of TWIST1 contributes to melanoma cell migratory deficits resulting from FOXD3 expression.

Materials and Methods

Cell Culture

Human melanoma cell lines WM115, WM793, and 1205Lu were donated by Dr. Meenhard Herlyn (Wistar Institute, Philadelphia, PA). TR versions express the Tet repressor. These cell lines were cultured in “WM medium”: MCDB 153 medium containing 20% Leibovitz L-15 medium, 2% FBS, 0.2% sodium bicarbonate, 5µg/ml insulin, and 1% penicillin/streptomycin (P/S). A375 cells were purchased from ATCC (Manassas, Virginia) and were grown in DMEM medium with 10% FBS and 1% P/S. The 293FT cell line was purchased from Invitrogen (Carlsbad, CA) and maintained in DMEM with 10% FBS, 1% P/S, and 1% non-essential amino acids. All melanoma cell lines used were verified as harboring BRAF mutations by DNA sequencing. Human melanocytes were isolated from neonatal foreskin samples obtained from elective circumcisions performed at the Obstetrics Unit at Thomas Jefferson University (22).

Lentiviral Construction and Transduction

The origins of the cloned genes used for overexpression are as follows: human TWIST1 (amplified from genomic DNA), human FOXD3 (amplified from genomic DNA), and β-galactosidase (LacZ; amplified from pLenti4/TO/V5-GW/LacZ (Invitrogen, Carlsbad, CA)). Sequences were cloned into pENTR/D-TOPO (Invitrogen) and recombined into a destination vector with either a puromycin or zeocin resistance cassette. Viral particles and cell lines were generated as in (23). The generation of Tet Repressor (TR)-expressing cells has been previously described (16). Transgene expression was induced by the addition of 100ng/ml doxycycline (Fisher Scientific, Fair Lawn, NJ) to the medium.

Inhibitors

PLX4720 was purchased from Selleck Chemicals (Houston TX).

Migration Assay

Sub-confluent melanoma cells were cultured for 24 hours in serum-free medium with 100ng/ml doxycycline. Uncoated 8.0µm pore-size cell culture inserts (BD Biosciences, San Jose, CA) were utilized. 5.0×10^4 cells in serum-free medium plus doxycycline were placed inside each chamber while full growth medium containing doxycycline was placed outside the chambers. Cells were allowed to migrate for 24 hours towards the attractant of full-serum medium. Chamber filter processing and visualization/quantitation of migration was performed, as previously described (23). Cell counts were converted to percent migration of the LacZ control (100%).

EdU Incorporation Assay

Sub-confluent melanoma cells were cultured for 48 hours in serum-free medium with 100ng/ml doxycycline to turn on transgene expression. For the final 7 hours, cells were

cultured in fresh medium containing 10 μ M EdU. After a PBS wash, the Click-iT EdU Alexa Fluor® 647 Flow Cytometry Assay Kit (Invitrogen) was utilized as per the manufacturer's instructions. Flow cytometry sorting was carried out at the Flow Cytometry Core Facility at Thomas Jefferson University and analyzed using FlowJo software.

Dual-Luciferase Assay

1205LuTR cells and A375TR cells engineered to overexpress either LacZ control or FOXD3 were transfected with Mirus *TransIT*-X2 (Mirus Bio LLC, Madison, WI). For psiCheck-2 vectors (Promega, Madison, WI), 1 μ g of each vector (empty, TWIST1 +909/+2135, or TWIST1 +2241/+3144) were transfected. For pTal-Luc vectors (Clontech, Mountain View, CA), 50ng pRL-TK Renilla vector and 1 μ g of either pTal-Luc empty vector or pTal-Luc/TWIST1 intron 1 vectors were transfected. At the time of transfection, cells were also treated with 100ng/ml doxycycline to induce expression of the transgenes. After 24 hours, luciferase assays were performed with the Dual-Luciferase Reporter Assay kit (Promega) according to manufacturer's instructions. Readings were taken using a Glomax 20/20 Luminometer (Promega). Results are expressed as fold change, which was calculated by dividing the normalized luciferase activities of FOXD3-overexpressing cells by the normalized luciferase activity of LacZ-overexpressing cells. Four independent experiments were performed.

Chromatin immunoprecipitation (ChIP) and ChIP-seq

ChIP-seq and analysis was performed previously (4). For validation of ChIP-seq, WM115TR-FOXD3-V5 cells were treated with 100ng/ml doxycycline for 24 hours and ChIP was performed as in (4). Four independent experiments were performed. ChIP-seq results were confirmed by performing quantitative PCR with primers within enrichment regions of the *TWIST1* locus. ChIP primer sets can be found in Supplementary Table S1.

Western Blot Analysis

Western blots were performed as previously described (23). Primary antibodies used: TWIST1 and B-RAF from Santa Cruz Biotechnology (Santa Cruz, CA); Actin from Sigma (St. Louis, MO); V5 epitope from Invitrogen; ERK1/2, phospho-ERK1/2, p21^{Cip1} from Cell Signaling Technology (Danvers, MA); FOXD3 from Biolegend (San Diego, CA). Chemiluminescence was visualized on a Versadoc MultiImager and quantitated with Quantity One Software (BioRad, Hercules, CA).

siRNA Transfection

WM793 and WM115 cells were transfected for 4 hours with chemically synthesized siRNAs (Dharmacon, Lafayette, CO) at a final concentration of 25nM using Oligofectamine (Invitrogen). Transfections were harvested at 96 hours. siRNA sequences are listed in Supplementary Table S1.

Quantitative RT-PCR

RNA was extracted from cells using RNeasy Plant Mini Kit (Qiagen, Valencia, CA) as per the manufacturer's instructions. Conversion to cDNA was achieved through the iScript

cDNA Synthesis Kit (Biorad). Quantitative RT-PCR was carried out using iQ SYBR Green Supermix (Biorad), 0.4 μ M oligonucleotide primers, and 0.1 μ g cDNA. Primer sets can be found in Supplementary Table S1. Relative fold change in mRNA levels were calculated after normalization to β -Actin using the comparative C_t method (24).

Statistical Analysis

Statistical analysis was performed using a two-tailed Student's t test. A p value < 0.05 was considered statistically significant.

Results

The stemness factor FOXD3 directly binds the TWIST1 locus at multiple locations

FOXD3 ChIP-seq analysis was previously undertaken by our laboratory (4). Briefly, human mutant BRAF WM115TR melanoma cells were induced to express V5-epitope tagged FOXD3 for 48 hours prior to processing in a standard ChIP-seq protocol. To determine whether TWIST1 is a direct target of FOXD3, ChIP-seq data was mined for enrichment of the *TWIST1* locus. Immunoprecipitation of the V5-epitope tag enriched chromatin encompassing regions in *TWIST1* intron 1/exon 2 and in the distal 3' untranslated region (UTR) (Figure 1A). Notably, *TWIST1* intron 1 contains a putative FOXD3 consensus binding site (AAACAAATGTT) that falls within the enriched region.

To confirm FOXD3 binding directly to the *TWIST1* locus, we performed additional ChIP experiments on WM115TR cells, which were untreated (no doxycycline) or were induced to express FOXD3-V5 for 24 hours. We then performed quantitative PCR with primers generated within *TWIST1* intron 1 or *TWIST1* 3'UTR (Figure 1B & C).

Immunoprecipitations with either a normal mouse IgG antibody or a V5-tag antibody from untreated WM115TR-FOXD3-V5 cells were used as negative controls. As expected, FOXD3-V5 immunoprecipitation significantly enriched the control RPL30 locus (data not shown). Importantly, both intron 1 and the distal 3'UTR of the *TWIST1* locus were significantly enriched by FOXD3 immunoprecipitation (Figure 1B & C; *p<0.05).

FOXD3 transcriptionally represses TWIST1

To determine the relevance of FOXD3 binding to the *TWIST1* locus, we examined four mutant BRAF melanoma cell lines for protein changes downstream of ectopic expression of FOXD3-V5 over a period of 3 days. FOXD3, which has the ability to act as a transcriptional activator or repressor, increased p21^{Cip1}/CDKN1A expression (Figure 2A), as has been previously described (16). Importantly, ectopic FOXD3 expression led to a marked decrease in TWIST1 protein expression over time in each of the cell lines tested. Examination of mRNA levels in these cell lines revealed a similar decrease in TWIST1 mRNA associated with FOXD3 overexpression (Figure 2B; *p<0.05, **p<0.01, ***p<0.001).

We next tested the ability of a knockdown of endogenous FOXD3 to enhance TWIST1 levels with two different siRNA targeting sequences. Low steady-state levels of FOXD3 in melanoma cell lines have previously been reported (16). As a positive control, FOXD3 was up-regulated by treatment with the RAF inhibitor, PLX4720, for 24 hours (16). TWIST1

protein expression was increased after endogenous FOXD3 knockdown in mutant BRAF WM793 and WM115 cells (Figure 2C and 2D). These data were supported by quantitative RT-PCR demonstrating that TWIST1 mRNA expression was significantly increased after FOXD3 knockdown (Figure 2E; * $p < 0.05$, ** $p < 0.01$). Similar data were observed in 1205Lu cells (Supplementary Figure S1). From these experiments, we conclude that endogenous FOXD3 is able to repress TWIST1 protein expression through reduction of TWIST1 mRNA.

TWIST1 is a member of a large family of EMT-promoting transcription factors. These factors can cooperate and/or have redundant roles in altering cell morphology and migration and are often altered concomitantly as part of an EMT program. For that reason, we sought out to determine whether FOXD3 overexpression altered other EMT proteins in addition to TWIST1. Importantly, FOXD3 was unable to consistently affect other EMT-promoting components, such as Slug, Zeb1 and Snail, across multiple cell lines (Supplementary Figure S2A). Thus, the FOXD3-dependent regulation of TWIST1 appears to be specific and not a product of a more global impact of FOXD3 on an EMT phenotype.

To further investigate the link between FOXD3 binding of the *TWIST1* locus and its impact on TWIST1 transcriptional repression, we performed dual-luciferase assays. Both enriched regions of *TWIST1* in the FOXD3 ChIP-seq were located downstream of the *TWIST1* coding region (Figure 3A). Therefore, vectors were constructed with *TWIST1* intron 1/exon 2 or *TWIST1* 3'UTR downstream of a luciferase cassette to determine whether FOXD3 could repress luciferase transcription in conjunction with these elements (Figure 3B). Transgene overexpression and endogenous TWIST1 reduction downstream of FOXD3 were confirmed by western blot (Figure 3D). Overexpression of FOXD3, when normalized to a LacZ control, was able to significantly reduce luciferase output when *TWIST1* regions were present downstream in two cell lines (Figure 3B; * $p < 0.05$, ** $p < 0.01$).

Often, intronic regions contain enhancer elements that can greatly influence transcription (4, 25, 26). In addition, the only apparent FOXD3 consensus site in either FOXD3-enriched *TWIST1* region was located in intron 1. Therefore, we examined whether intron 1 of *TWIST1* contains enhancer elements blocked by FOXD3 binding. Luciferase vectors were constructed to include the *TWIST1* intron 1 upstream of a minimal promoter linked to a luciferase cassette (Figure 3C). As expected, FOXD3 decreased endogenous TWIST1 protein levels in A375TR cells (Figure 3D). Importantly, FOXD3 overexpression reduced luciferase output downstream of *TWIST1* intron 1 (Figure 3C; * $p < 0.05$). In summary, the binding of FOXD3 to regions of the *TWIST1* locus directly reduces TWIST1 transcription, possibly through the blockade of enhancer elements.

TWIST1 promotes migration in FOXD3-overexpressing cells

Previous studies have established that changes in FOXD3 and TWIST1 expression can alter cell migratory capabilities in both developmental and cancer contexts (17, 23, 27-32). To determine whether TWIST1 expression could reverse defects in migration due to FOXD3 overexpression, 1205LuTR and A375TR cells were engineered to inducibly express either a LacZ or a FOXD3 gene cassette, with or without TWIST1 overexpression. Transgene induction by 24 hours of doxycycline treatment was confirmed by western blot analysis

(Figure 4A & C). Following transgene expression, cells were assessed for their ability to migrate in a transwell migration assay for 24 hours. As expected from previous studies, FOXD3 overexpression alone significantly ablated the ability of melanoma cells to migrate as compared to LacZ overexpression in 1205Lu and A375 cells (Figure 4B & D; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). TWIST1 overexpression in a LacZ background resulted in an increase of migration over baseline (Figure 4B & D; * $p < 0.05$). Notably, TWIST1 re-expression in FOXD3 overexpressing cells was able to partially (A375TR) or fully (1205LuTR) reverse the migration deficit of FOXD3-expressing cells (Figure 4B & D, ** $p < 0.01$, *** $p < 0.001$). Therefore, TWIST1 promotes migration in FOXD3-expressing melanoma cells.

Next, we determined whether TWIST1 counteracted FOXD3-induced effects on cell cycle progression. FOXD3 expression is known to up-regulate the cyclin-dependent kinase inhibitor, p21^{Cip1}, which impairs melanoma cell growth through the induction of G₁ cell cycle arrest (16). We therefore examined the S-phase profile of 1205TR and A375TR cells after 48 hours of transgene induction, the same conditions utilized for migration assays. FOXD3 overexpression significantly reduced the number of cells in S-phase in both cell lines compared to control (Figure 4E & F; * $p < 0.05$, ** $p < 0.01$). In the 1205LuTR cell line, TWIST1 overexpression slightly increased the population of cells in S-phase as compared to LacZ expression alone and to FOXD3 expression alone (Figure 4E). In A375TR cells, TWIST1 had no effect on S-phase entry (Figure 4F). Alterations in p21^{Cip1} protein expression mirrored the differences in S-phase profile. TWIST1 overexpression had a greater effect on reduction of p21^{Cip1} levels than LacZ or FOXD3 expression in 1205LuTR cells but TWIST1 expression did not alter p21^{Cip1} in A375TR cells (Figure 4A & C).

The lack of p21^{Cip1} changes in A375TR cells downstream of TWIST1 prompted us to explore the regulation of proteins important in the migratory cascade. We analyzed phosphorylation of the actin severing protein cofilin that is downstream of Rho family GTPases. In A375TR cells, TWIST1 increased phosphorylation of cofilin, which is indicative of active Rho family GTPase pro-migratory signaling (Figure 4C). Altered cofilin phosphorylation was also apparent, although to a lesser extent, in 1205LuTR cells (Figure 4A). Taken together, these results indicate that FOXD3 inhibits cell migration via down-regulation of TWIST1, although the mode of TWIST1 action may be cell-context dependent.

Discussion

Our results indicate that TWIST1 is a novel target of FOXD3 in melanoma. FOXD3 directly binds to the *TWIST1* gene locus at several locations and represses *TWIST1* transcription. Previous interrogation of the patterns of FOXD3-bound chromatin revealed that a large percentage of all binding regions were intronic, including the first intron of the *ERBB3* gene (4). Indeed, FOXD3 was able to repress luciferase output both when the intronic region of *TWIST1* was downstream of a luciferase-coding cassette, as it is in the *TWIST1* endogenous locus, as well as positioned upstream in a typical “enhancer” site. The intronic region also contains a putative FOXD3 consensus binding sequence. Notably, the transcription factor DLX4 has been found to increase TWIST1 transcription by directly binding to several

regions in the *TWIST1* locus, including a region adjacent to intron 1 (31). It is intriguing to postulate that a primary role of FOXD3 in melanocytic cells is to affect the timing of expression of TWIST1 through direct binding of chromatin and the blockade of DLX4 or other transcriptional activators.

The temporal interplay between FOXD3 and TWIST1 could greatly impact melanocyte and melanoma cell biology. Both proteins play roles in cell motility, a key step in developmental processes such as gastrulation and in the progression of tumor cells towards metastatic spread (17, 23, 27-32). We have previously shown that FOXD3 inhibits melanoma cells migration and invasion and TWIST1 promotes invasive properties through 3D collagen (17, 23, 27-32). Here, we show that ectopic expression of TWIST1 is able to counteract deficits in melanoma cell migration brought about by FOXD3 overexpression. Regulation of cell motility also involves Rho family GTPases, Rho, Rac, and Cdc42, and the LIM kinase-cofilin pathway that control lamellipodium formation, invadopodia dynamics, and actin severing (33-35). Previous work revealed that TWIST1, through regulation of the microRNA let-7i, is able to increase Rac1 activation in head and neck squamous cell carcinoma (36). Similarly, microRNA-10b, a known TWIST1 target, leads to increased RhoA and/or RhoC protein expression in human glioma and breast cancer cells as well as increased migration and invasion (37-39). In our melanoma studies, ectopic expression of TWIST1 in both A375 and 1205Lu cells increased phosphorylation of cofilin, indicative of active Rho/Rac/Cdc42 signaling, although the effect was relatively modest in 1205Lu cells. TWIST has also been shown to regulate multiple other migration- and invasion-related targets (23, 40).

In 1205Lu cells, TWIST1 overexpression also reduces p21^{Cip1} protein expression in both basal (LacZ control) and FOXD3-mediated up-regulated p21^{Cip1} backgrounds. Alterations in p21^{Cip1} levels in 1205Lu cells correlate with changes, albeit minor, in S-phase entry. In addition, previous studies have found that cytoplasmic p21^{Cip1} can alter the migratory capacity of cells through its effects on the actin cytoskeleton and focal adhesions (41, 42). TWIST1 has been shown in a number of contexts to regulate p21^{Cip1} expression, particularly in non-small cell lung cancer, sarcomas, and mesenchymal stem cells. TWIST1 cooperates with K-Ras^{G12D} in lung tumorigenesis through suppressing key drivers of cell senescence such as p21^{Cip1}, p16^{INK4A}/CDKN2A, and p27^{Kip1}/CDKN1B (43). Sarcomas, derived from mesenchymal cells, as well as mesenchymal stem cells also display TWIST1 regulation of p21^{Cip1} by both p53-dependent and -independent mechanisms (44-46). The inability of overexpressed TWIST1 to abrogate p21^{Cip1} up-regulation as a result of FOXD3 expression in A375 cells may be associated with the high p53 steady state levels in these cells (Supplementary Figure S2B).

In summary, we describe a novel interplay between two neural crest transcription factors, FOXD3 and TWIST1, and their opposing effects on the migration and invasive properties of mutant BRAF melanoma cells. The conversion between FOXD3 and TWIST1 expression states may control the acquisition of traits required to promote distinct steps within the metastatic cascade, independent of new mutations. Given the strong evidence of heterogeneity within melanoma (47, 48) indicating functionally distinct states, defining

markers for these states and identifying mechanisms of inter-conversion between these states are important future goals.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Implications

FOXD3 and TWIST1 define distinct sub-groups of cells within a heterogeneous tumor.

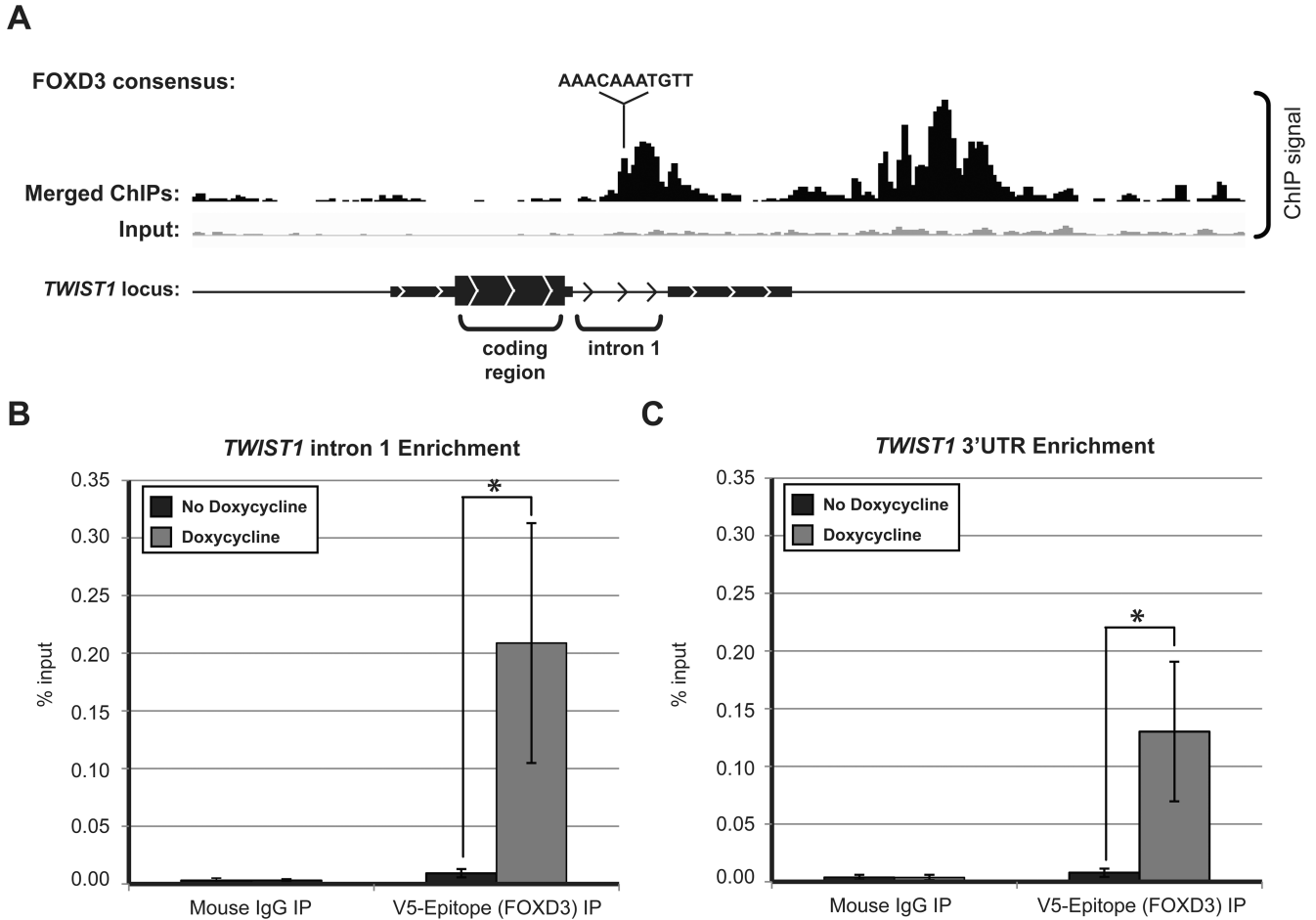
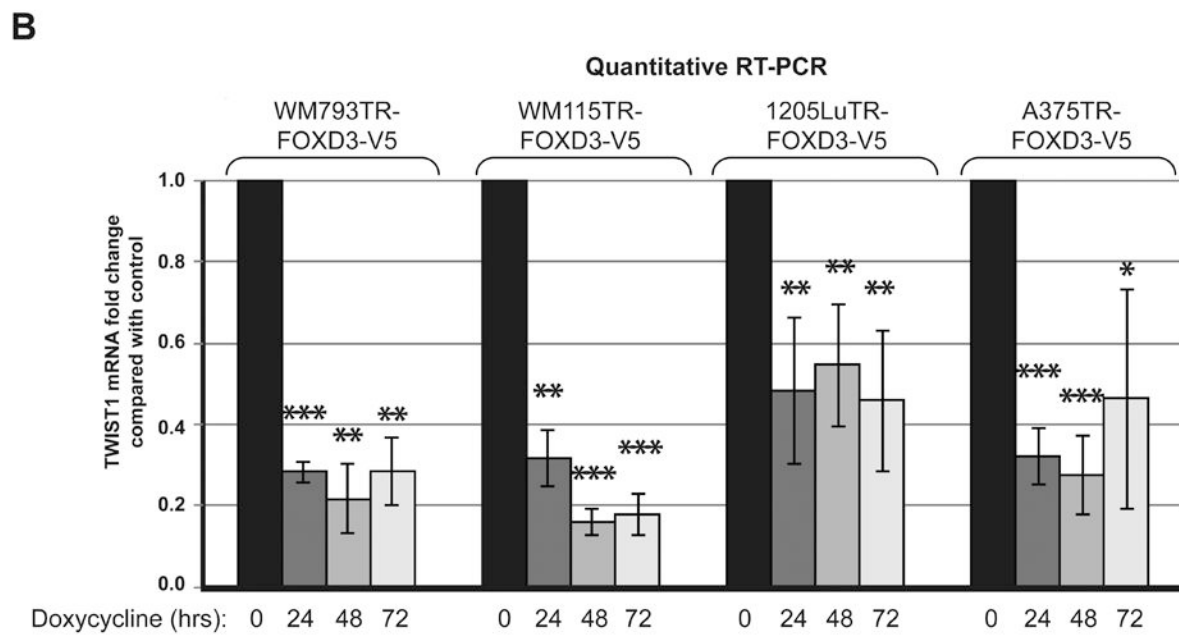
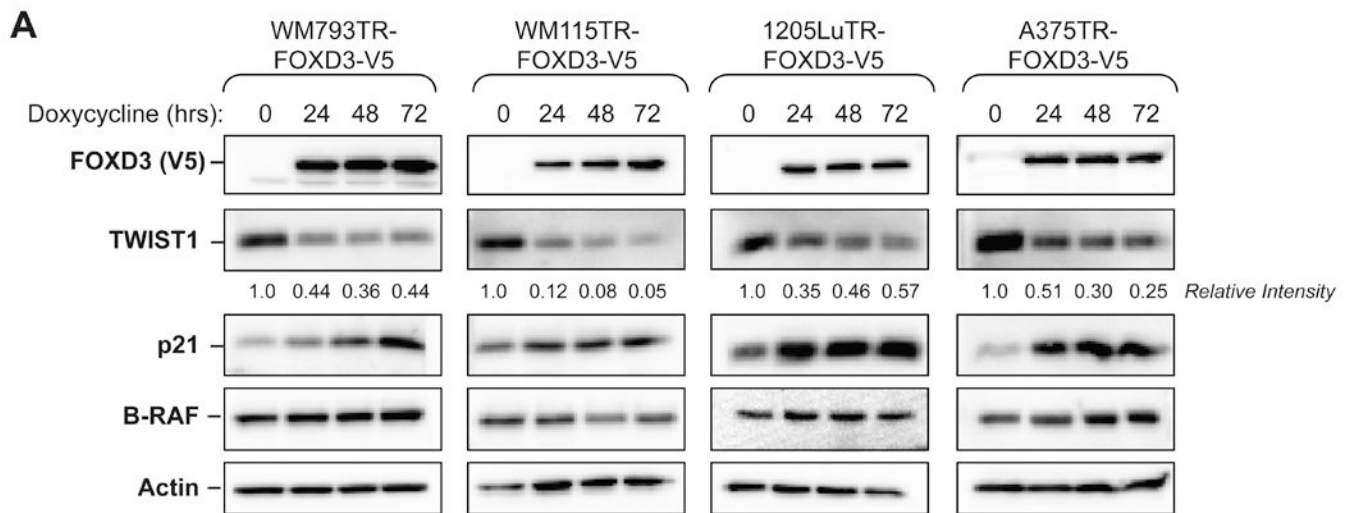
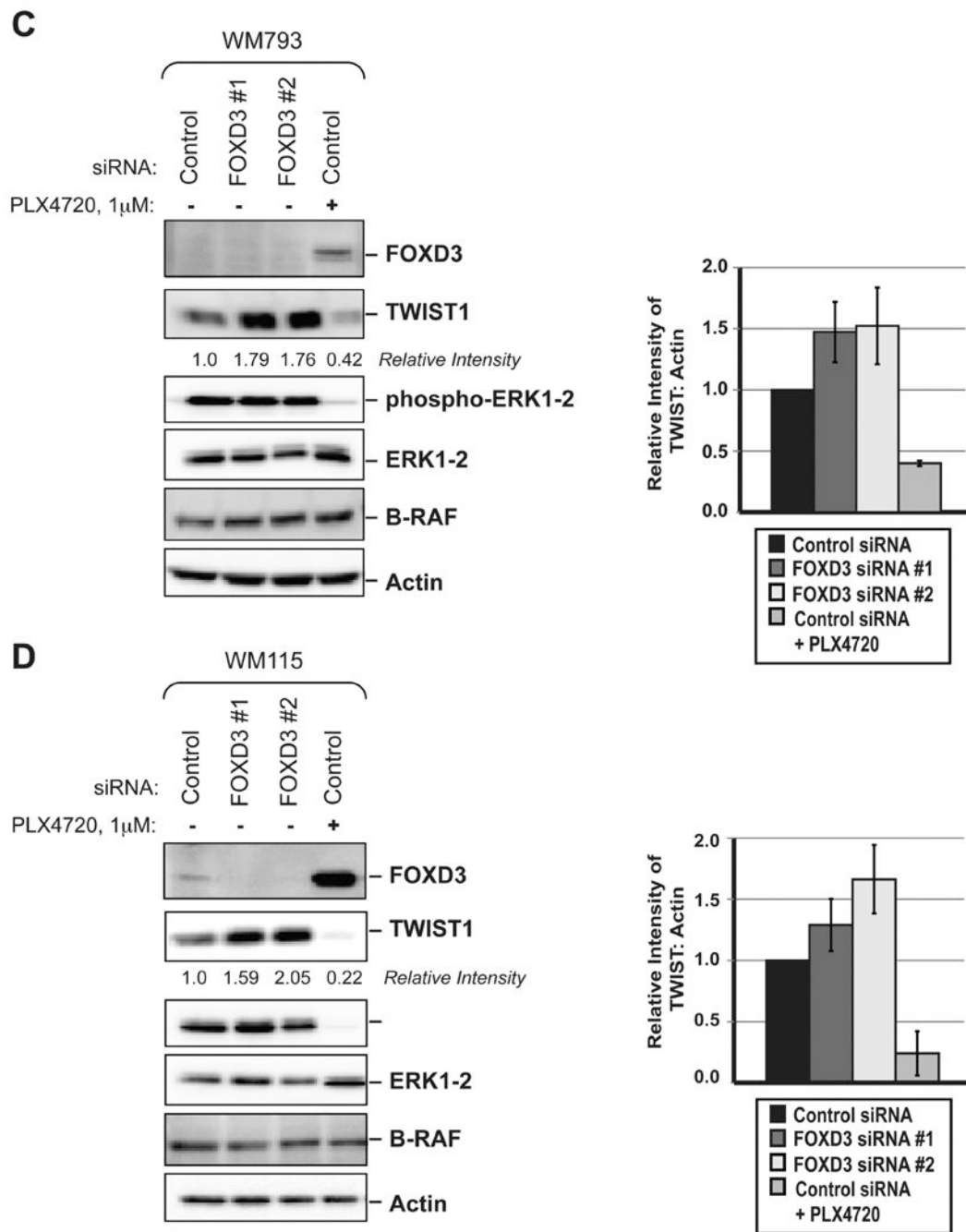


Figure 1. The stemness factor FOXD3 directly binds the *TWIST1* locus at multiple locations (A) FOXD3 ChIP-seq analysis was previously undertaken by our laboratory. WM115TR-FOXD3-V5 cells were induced to express FOXD3-V5 for 48 hours with 100ng/ml doxycycline or left untreated followed by ChIP sequencing (TR= Tet Repressor). FOXD3-bound chromatin was purified with a V5-epitope tag antibody. ChIP with mouse IgG antibody was used as a negative control. These data were mined for enrichment of *TWIST1* locus regions by FOXD3 binding. Shown is a map of the *TWIST1* locus showing read coverage for IP and input. Light gray peaks are representative of the signal from merged inputs, while the black peaks represent the relative signal of merged ChIP experiments. (B and C) Regular ChIP samples of WM115TR-FOXD3-V5 cells +/- 24 hours of doxycycline were generated to confirm ChIP-seq data. Enrichment of *TWIST1* intron 1 (B) and 3'UTR (C) was validated by quantitative PCR. Columns, average of four independent experiments; bars, SD; * $p < 0.05$.





E

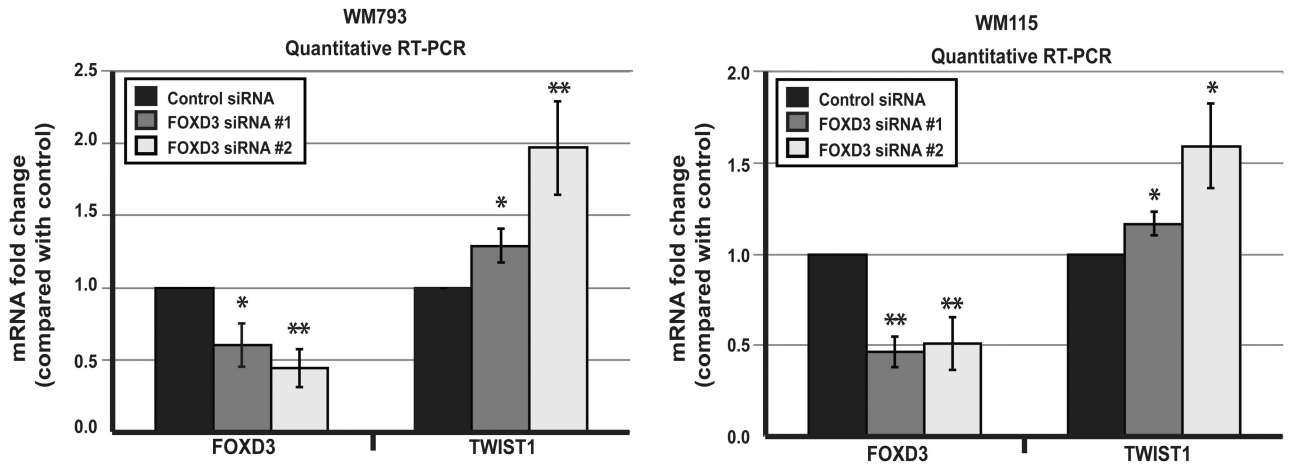


Figure 2. FOXD3 transcriptionally represses TWIST1

(A) WM793TR, WM115TR, 1205LuTR, and A375TR cells were transduced with an inducible V5-epitope-tagged FOXD3 gene cassette. At 0, 24, 48, and 72 hours of induction with doxycycline (100ng/ml), lysates were harvested and western blots were performed. (B) Similar to (A), time courses of FOXD3 expression were performed and RNA isolated at each time point. Quantitative RT-PCR was performed. Columns, average of four independent experiments; bars, SD; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. (C) WM793 cells or (D) WM115 cells were transfected with a control non-targeting siRNA or one of two different FOXD3 targeting siRNAs. Lysates and mRNA were harvested four days after siRNA transfection. In addition, control siRNA treated cells were treated for 24 hours with the RAF inhibitor PLX4720 (1 μ M) as a positive control for FOXD3 expression. Western blots and quantitation performed using Versadoc imaging from three independent experiments. Bar, SD. (E) As for C and D, except that RT-PCR was performed. Columns represent the average of three independent experiments; bars, SD; * $p < 0.05$, ** $p < 0.01$.

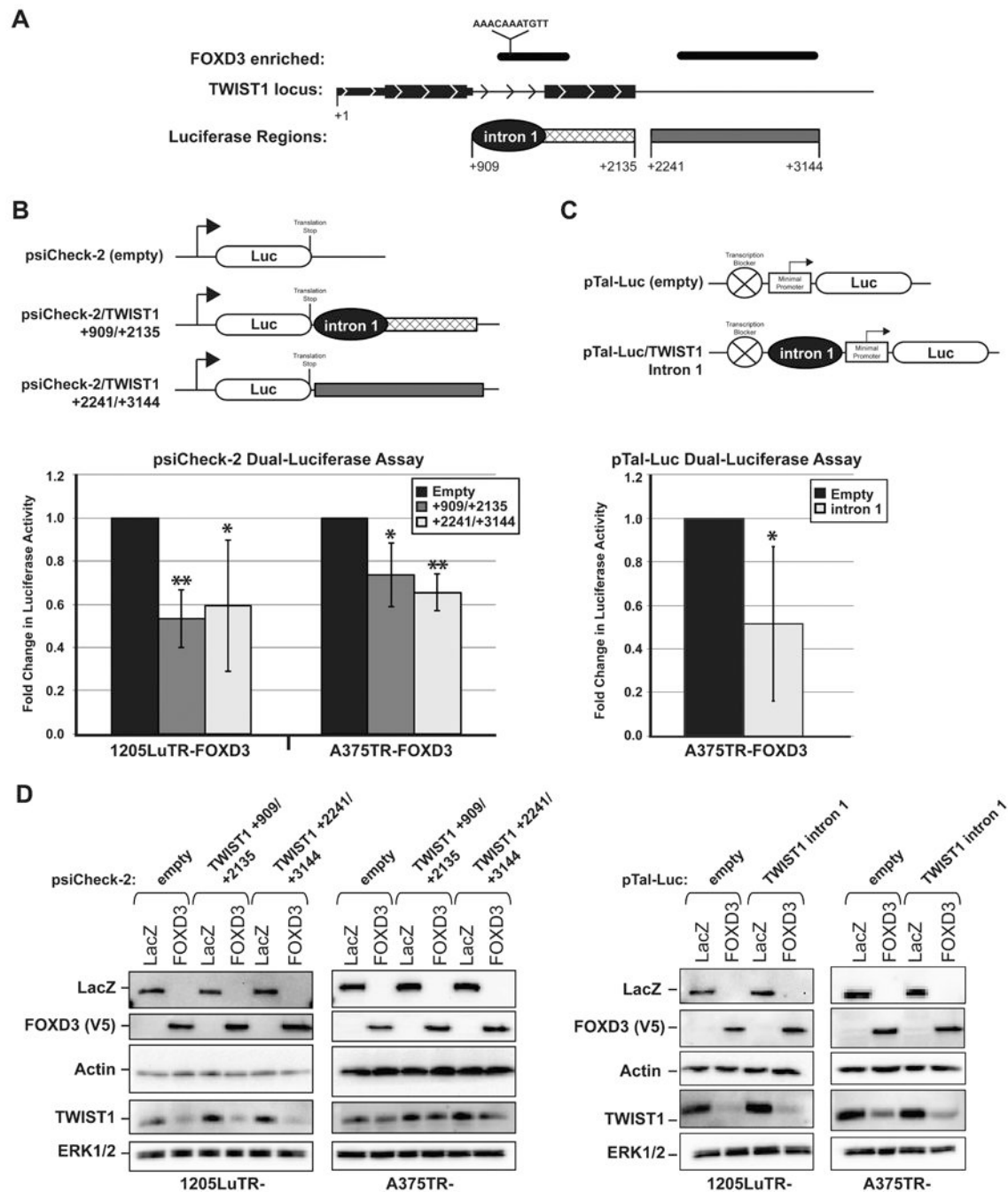


Figure 3. FOXD3 alters luciferase output downstream or upstream of TWIST1 locus elements (A) A schematic of the *TWIST1* locus highlighting the regions enriched in the FOXD3 ChIP-seq with potential consensus sequences present. Indicated below are the regions utilized in the dual-luciferase assays. (B) A dual-luciferase assay was performed to determine whether the FOXD3 expression would alter luciferase output with TWIST1 locus elements cloned downstream of luciferase. Fold change was calculated by dividing the normalized luciferase activities of FOXD3-expressing 1205TR or A375TR cells by the normalized luciferase activity of LacZ-expressing cells. Columns, average of four independent experiments; bars,

SD; * $p < 0.05$, ** $p < 0.01$. (C) Dual-luciferase assays were performed as in (B) but with TWIST1 intron 1 cloned upstream of luciferase under the control of a minimal promoter. Columns, average of four independent experiments; bars, SD; * $p < 0.05$. (D) Western blots confirmed differential expression of FOXD3 (V5-epitope tag) and LacZ within the luciferase experiments in (B & C).

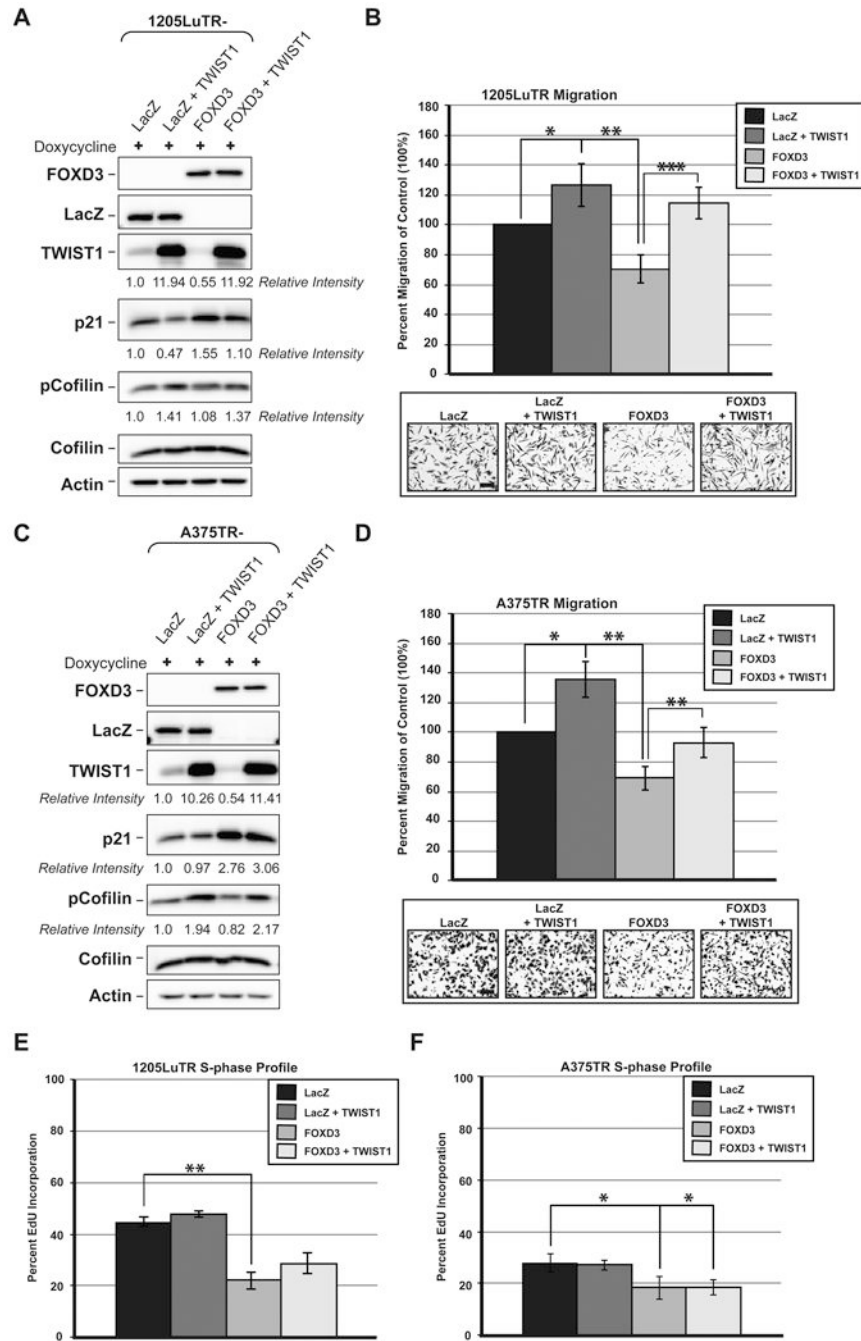


Figure 4. TWIST1 reverses defects in cell migration resulting from FOXD3 overexpression (A & B) 1205LuTR cells were engineered to express either a LacZ gene cassette or a FOXD3 gene cassette, with or without TWIST1 overexpression, upon doxycycline addition (100ng/ml). Cells were serum starved and induced with doxycycline for 24 hours prior to a Transwell migration assay. Western blot analysis was performed to confirm transgene expression. Cells were allowed to migrate towards an attractant of full serum medium (+ doxycycline) for 24 hours. Counts taken (in triplicate fields of view) from the LacZ overexpression (set at 100% migration) were used to calculate percent migration for all other

treatments. Average percent migration from 3 independent experiments is displayed. One representative field of view is shown; scale, 100 μ m. Columns, average of three independent experiments; bars, SD; *p<0.05, **p<0.01, ***p<0.001. (C & D) A375TR cells were assayed as in (A & B). Columns, average of three independent experiments; bars, SD; *p<0.05, **p<0.01. (E & F) Cells were subjected to a 7 hour EdU incorporation at the end of a 48 hour serum-free with doxycycline treatment. S-phase profiles were analyzed by flow cytometry. Percent EdU incorporation of the total cell population is displayed. Columns, average of three independent experiments; bars, SD; *p<0.05, **p<0.01.