The Twist Box Domain Is Required for Twist1-induced Prostate Cancer Metastasis

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Abstract

Twist1, a basic helix-loop-helix transcription factor, plays a key role during development and is a master regulator of the epithelial–mesenchymal transition (EMT) that promotes cancer metastasis. Structure–function relationships of Twist1 to cancer-related phenotypes are underappreciated, so we studied the requirement of the conserved Twist box domain for metastatic phenotypes in prostate cancer. Evidence suggests that Twist1 is overexpressed in clinical specimens and correlated with aggressive/metastatic disease. Therefore, we examined a transactivation mutant, Twist1-F191G, in prostate cancer cells using in vitro assays, which mimic various stages of metastasis. Twist1 overexpression led to elevated cytoskeletal stiffness and cell traction forces at the migratory edge of cells based on biophysical single-cell measurements. Twist1 conferred additional cellular properties associated with cancer cell metastasis including increased migration, invasion, anoikis resistance, and anchorage-independent growth. The Twist box mutant was defective for these Twist1 phenotypes in vitro. Importantly, we observed a high frequency of Twist1-induced metastatic lung tumors and extrathoracic metastases in vivo using the experimental lung metastasis assay. The Twist box was required for prostate cancer cells to colonize metastatic lung lesions and extrathoracic metastases. Comparative genomic profiling revealed transcriptional programs directed by the Twist box that were associated with cancer progression, such as Hoxa9. Mechanistically, Twist1 bound to the Hoxa9 promoter and positively regulated Hoxa9 expression in prostate cancer cells. Finally, Hoxa9 was important for Twist1-induced cellular phenotypes associated with metastasis. These data suggest that the Twist box domain is required for Twist1 transcriptional programs and prostate cancer metastasis.

Implications: Targeting the Twist box domain of Twist1 may effectively limit prostate cancer metastatic potential.

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Introduction

Prostate cancer is the most common cancer diagnosed in men in the United States and is responsible for the second most cancer deaths in men (1). Patterns of disease failure in prostate cancer suggest understanding the determinants that confer progression of localized presentations to metastatic disease will result in the largest therapeutic gains (2).

One mechanism by which cancer cells may acquire the characteristics necessary for metastasis is the epithelial–mesenchymal transition (EMT). EMT is a transcriptional program, crucial in early embryonic development that is co-opted by some cancer cells to facilitate aggressive and metastatic behavior (3). Twist1 is a basic helix-loop-helix (bHLH) multidomain transcription factor which directly mediates EMT by transcriptional activation and repression of E-box-regulated target genes (4, 5). A role for Twist1 in prostate cancer pathogenesis has been suggested (6, 7), but the role of EMT and Twist1 in prostate cancer disease...
progression and metastasis is just now being explored (8, 9). The critical domains of Twist1 and the crucial Twist1 downstream transcriptional targets required for increased tumorigenicity and aggressive metastatic phenotypes in prostate cancer are unknown. The carboxyl-terminal Twist box is a highly conserved domain among Twist1 orthologues for which little functional information in the context of cancer phenotypes is known (5). A greater understanding of the structure–function relationships and downstream targets of Twist1 may allow for an increased appreciation of the mechanisms responsible for Twist1-induced metastasis and may facilitate more precise inhibitory strategies of Twist1 as a therapeutic maneuver in cancer.

Here, we used a single amino acid substitution mutation, Twist1 codon 191 phenylalanine-to-glycine (F191G), to study the role of the Twist box for Twist1-induced aggressive cellular and metastatic phenotypes in prostate cancer cells. Isogenic androgen-dependent, Myc-CaP (10), and androgen-independent, PC3, cell lines overexpressing Twist1 or the Twist box mutant showed specific requirements for the Twist box during Twist1-induced metastasis of prostate cancer cells. Gene expression profiling revealed transcriptional programs directed by the Twist box that were associated with cancer metastasis. Finally, we show that Twist1 directly regulates one such target, Hoxa9, which is partially required for Twist1-induced prostate cancer prometastatic phenotypes.

Materials and Methods

Plasmids, antibodies, and reagents

pBABE-Twist1-puro or –hygro (11) was used to construct the Twist1-F191G mutant using the QuikChange Site-Directed Mutagenesis Kit (Stratagene) and confirmed by sequencing. Antibodies used were: Twist (Twist2C1a; sc-81417, Santa Cruz Biotechnology), E-cadherin (ab53033, Abcam), vimentin (ab92547), ZO-1 (5406, Cell Signaling Technology), β-actin (A5316, Santa Cruz Biotechnology), c-Myc (N-term; 1472-1, Epitomics), horseradish peroxidase-conjugated secondary antibodies (Invitrogen), and Alexa Fluor 488 conjugated secondary antibodies (Invitrogen). Hoxa9 shRNA retroviral constructs were purchased and used as directed by Origene (cat #TG500979).

Cell line and culture conditions

PC3 and 22RV1 were obtained from American Type Culture Collection. Myc-CaP was a kind gift from Dr. John Isaacs (Johns Hopkins University, Baltimore, MD; ref. 10). Growth media: Myc-CaP, Dulbecco’s modified Eagle medium (Invitrogen); PC3, Hams F12K (Invitrogen); and 22RV1, RPMI-1640 (Invitrogen). Cell line identity confirmed by short tandem repeat profiling and mycoplasma tested. All media were supplemented with 10% FBS and penicillin (100 U/mL), streptomycin (0.1 mg/mL). Cells were maintained at 37°C in a humidified incubator with 5% CO2 in air.

Retroviral experiments

Retroviral production used ecotropic and amphotropic Phoenix packaging lines. Myc-CaP cells were transduced with pGFP-V-RS-based shRNA constructs from Origene as described above or with scrambled control vector for two successive times over a 36-hour period followed by selection with 1 mg/mL puromycin and passaged once 80% confluent.

Luciferase promoter reporter assay

Subconfluent cells were transfected using Lipofectamine 2000 (Invitrogen) with 200 ng of firefly luciferase reporter gene construct (100 ng was used for SNAI2 reporter assays), 100 ng of the pRL-SV40 Renilla luciferase construct, and 500 ng of the Twist1 or Twist1-F191G–mutant expression construct. Cell extracts were prepared 36 hours after transfection in passive lysis buffer, and the reporter activity was measured using the Dual-Luciferase Reporter Assay System (Promega).

Wound-healing migration assay

Two-dimensional migration assay was conducted using a scratch/wound model. Cells were grown in 6-well plates for 24 hours to confluence. PC3 cells were treated with 500 pmol/L TGF-β at the time of wounding. Multiple scratch wounds were created using a P-20 micropipette tip and cells fed with fresh complete media. Five representative fields of the wound were marked and images were taken at 0 and 24 hours after wounding. Relative wound closure is calculated from the remaining wound area normalized to the initial wound area using ImageJ software (NIH Image).

Biophysical assays

Fourier transform traction microscopy (FTTM) was used to measure the contractile stress arising at the interface between each adherent cell and its substrate as described (12). Briefly, cells were plated sparsely on elastic collagen type I coated gel blocks. Images of fluorescent microbeads (0.2 μm in diameter, Molecular Probes) embedded near the gel apical surface was taken at different times with cell-free reference (traction-free) images. The displacement field between a pair of images was then obtained by identifying the coordinates of the peak of the cross-correlation function (13, 14). From the displacement field and known elastic properties of the gel (Young’s modulus of 1 kPa with a Poisson’s ratio of 0.48), the cell traction field was computed. The computed traction field was used to obtain net contractile moment, which is a scalar measure of the cell’s contractile strength, expressed in pico-Newton meters (pNm).

Magnetic twisting cytometry (MTC) was used to measure material properties of the cytoskeleton as described (15, 16). In brief, cells were plated at 150,000 cells/cm2 on coated collagen type I plastic wells (96-well Removawell, Immunol II; Dynetech) at 500 ng/cm2. After scratching with a 200-μL pipette tip and the indicated time, ferrimagnetic microbeads were functionalized to the cytoskeleton, and both stiffness, g, and loss modulus g′, were measured over a physiologic range of frequency (f) expressed in Pascal per nm (Pa/nm).

Matrigel invasion assay

The invasion potential was assessed using Chemicon cell invasion assay kit (Millipore) as directed by the
manufacturer. Of note, 8 μmol/L Transwells with Matrigel were used for the assay. Serum-starved 0.5 to 1 × 10⁶ cells (12–16 hours) in 300 μL were seeded in upper chambers, whereas lower wells were filled with 500 μL of 10% FBS complete medium. Invading cells on the lower surface were fixed and stained. The stain is dissolved in 200 μL of 10% acetic acid and measured at 570 nm. Invasive potential is derived by normalizing with the readings from blank Transwell inserts.

**Immunohistochemistry, Immunofluorescence, and Western blotting**

Immunohistochemistry (IHC), immunofluorescence, and Western blotting were conducted as described previously (17).

**Anoikis assay and apoptosis assessment**

Anoikis resistance was measured using a modified protocol (18). Cells were grown in normal attachment and ultra-low attachment (Corning) in 6-well plates. Twenty-four hours later, cells were blocked in 5% FBS and stained with Alexa Fluor 488 conjugated Annexin V followed by propidium iodide staining (50 μg/mL; Invitrogen). Cells were enumerated on a BD FACS Caliber (BD Biosciences), and analysis was done using FlowJo analysis software. All conditions were n = 4 and two replicates per experiment.

**Clonogenic survival and soft agar colony formation assays**

Clonogenic survival was conducted as previously described (19). Soft agar clonogenic assays used 6-well plates precoated with 1 mL of basal 0.6% agarose in complete media and overlaid with 2 mL of cells (5 × 10³ cells/mL) mixed with 0.3% agarose in complete media to solidify. The wells were constantly fed with complete media to prevent drying of agarose, and then after 10 to 15 days of incubation, colonies were scored under phase contrast microscopy. All conditions were repeated at least twice with 3 wells per experiment.

**Animal models and histology**

All procedures were carried out in accordance with the Johns Hopkins Animal Care and Use Committee, maintained under pathogen-free conditions, and given food and water ad libitum. For the subcutaneous tumor graft assay, 100 μL of PBS and Matrigel (BD Biosciences) mixed 1:1 containing 0.5 to 2 × 10⁶ cells were injected subcutaneously into both the flanks of 8-week-old male FVB/N or athymic nude mice. Tumors measured 3 times weekly and volume calculated: length × width × height × π/6. Tumor growth delay is the difference between the quadrupling times of untreated versus treated tumors. For the experimental lung metastasis assay, 100 μL of PBS containing 5 × 10⁵ cells were injected into athymic nude mice via the tail vein. After 4 weeks, the mice were sacrificed, necropsies conducted to score surface lung tumors and extrathoracic metastases.

**Microarray data acquisition and analysis**

Microarrays were conducted using GeneChip WT cDNA Synthesis and amplification Kit and WT terminal labeling Kit (Affymetrix). The labeled ssDNA was hybridized to the GeneChip Mouse Gene 1.0 ST array (Affymetrix), washed with the Fluidics station 450, and array scanning was conducted as previously described. Arrays were normalized using the Robust Multichip Average in the oligo Bioconductor package at the transcript level. Genes and gene sets with Benjamini–Hochberg P < 0.05 were considered statistically significant. Gene set enrichment analysis (GSEA) was conducted using the C2 Curated Gene Sets collection from the Molecular Signature Database 3.0 and statistical comparisons by Fisher Exact test. More detailed description of the analysis and R code used for this analysis are included as Supplementary Materials and Methods.

**Chromatin immunoprecipitation**

Chromatin immunoprecipitation (ChIP) was conducted using a SimpleChIP Enzymatic IP Kit (Cell Signaling Technology). See Supplementary Materials and Methods for details.

**SYBR-green quantitative RT-PCR and prostate cancer cDNA arrays**

The iTaq Universal SybrGreen Master Mix (BioRad) was used according to the manufacturer’s instructions. Human normal prostate and prostate cancer qPCR tissue arrays and TWIST1 qPCR oligos were purchased from OriGene. All relevant clinical information can be found in http://www.ogene.com/qPCR/Tissue-qPCR-Arrays.aspx.

**Statistical analysis**

Statistical analysis was carried out using GraphPad Prism v5.04 (GraphPad Software). Paired comparisons were tested using the Mann–Whitney test or Fisher exact test. Throughout this study: *P ≤ 0.05; **P ≤ 0.01; and ***P ≤ 0.001.

**Results**

**TWIST1 is overexpressed in prostate cancer and correlates with aggressive and metastatic disease**

**TWIST1** expression in prostate cancers was analyzed from 14 independent microarray datasets constituting 1,013 prostate samples (20–32) using Oncomine. Ten of the 14 datasets and the aggregate analysis showed **TWIST1** overexpression in prostate cancers (Fig. 1A, P = 0.002 for aggregate). Further analysis of one of these microarray studies (20) showed that **TWIST1** overexpression correlated with metastatic disease (Fig. 1B; P < 0.000001). This microarray data were validated using quantitative PCR (qPCR) for **TWIST1** on human prostate cancer samples. Cancer samples (n = 107) screened by qPCR confirmed that **TWIST1** was overexpressed in prostate cancer (40/107 or 37% showed ≥3-fold upregulation, 18/107 or 17% ≥10-fold overexpression, and some cases ≥50-fold overexpression; Fig. 1C; P < 0.0001). Similar to the microarray data, **TWIST1** overexpression was directly correlated with prostate cancer aggressiveness as determined by
Gleason score (Fig. 1D; \( P < 0.0001 \)). These data agreed with prior studies that showed \( \text{TWIST1} \) overexpression in human prostate cancer and correlation with prostate cancer disease aggressiveness and metastasis (7, 33).

**The Twist box domain is required for full transcriptional activity of Twist1 in prostate cancer cells**

Twist1 has four domains: (i) a Twist1 domain that is highly conserved among human and mice; (ii) a glycine rich region; (iii) bHLH domain; and (iv) the Twist box (or WR domain). The Twist box is identical between mouse, human, frog, zebra fish, and jellyfish species and is located in the last 23 residues of the mouse polypeptide. The Twist box has been shown to be necessary and sufficient to transactivate E-box containing heterologous reporter constructs \( \text{in vitro} \) (34). We generated a site-specific Twist box mutant by mutating a critical Phe-191 to Gly, referred to as Twist1-F191G (Fig. 2A). This Twist1-F191G mutant had...
Figure 2. The Twist box mutant is deficient for Twist1 transcriptional activity and displays an attenuated EMT cellular marker profile in prostate cancer cells. A, a schematic of Twist1 protein structure and the position 191 phenylalanine site-specific mutant examined (this schematic is not to scale). Key functional residues required for transcriptional transactivation in the Twist box, L-187, F-191, and R-195, are shown in green. We created constructs overexpressing the Twist1-F191G mutant which has the substitution of phenylalanine-191 for glycine. B, Twist1 promoter reporter assays show that the Twist1-F191G mutant is defective for transcriptional activity. Myc-CaP cells were transiently transfected with expression vectors for the firefly luciferase-linked human E-cadherin gene (CDH1) promoter construct and a Renilla luciferase reporter vector for normalization of transfection efficiency. After 36 hours, cell extracts were assayed for luciferase and Renilla activity and showed Twist1 overexpression repressed transcription from the E-cadherin gene promoter, but the Twist1-F191G mutant was attenuated for this function (***, P < 0.001; and *, P < 0.05 by Mann–Whitney test). Similar reporter assays were conducted using a Slug gene (SNAI2) promoter construct and showed that the Twist1-F191G mutant had no ability to transactivate transcription compared with wild-type Twist1 overexpression (***, P < 0.001 by Mann–Whitney test). Each bar represents values from five to six independent experiments conducted in triplicate. Bars represent column mean; error bars ±SEM. Western blot analysis was conducted for Twist1 expression in (C) Myc-CaP (left) and PC3 (right) cells stably expressing Vector and overexpressing similar levels of Twist1 or Twist1-F191G with β-actin used as a loading control. Epithelial and mesenchymal markers were also assessed by (C) Western blotting and (D) immunofluorescence for Twist1, E-cadherin, ZO-1, and vimentin in Myc-CaP (left) and PC3 (right) cells.
been shown previously to be deficient for transactivation of E-box containing reporter constructs in mesenchymal cells (34), but has not been examined in epithelial cancer cells. In Myc-CaP androgen-dependent prostate cancer cells, Twist1 overexpression significantly repressed CDH1 (Fig. 2B, left; \( P < 0.001 \)) promoter activity (35) and increased SNAI2 (Fig. 2B, right; \( P < 0.001 \)) promoter activity. Twist1-F191G was found to be defective for both repression and activation in these assays and significantly different from Twist1 (Fig. 2B; \( P < 0.05 \) for both). There was some suggestion that Twist1-F191G was more defective for activation than repression, as Twist1-F191G was still able to repress the CDH1 promoter to some extent but could not activate the SNAI2 promoter as compared with Vector control (Fig. 2B, left, \( P < 0.05 \) and right, \( P = 0.0952 \)). Similarly, in HEK 293 cells, Twist1 repressed CDH1 promoter activity, whereas Twist1-F191G only partially repressed the CDH1 promoter activity compared with Twist1 wild-type (Supplementary Fig. S1A, both \( P < 0.001 \)). Neither Twist1 nor Twist1-F191G seemed to alter expression from the SNAI2 promoter in HEK 293 cells, which is not surprising as this cell line is of likely mesenchymal origin (Supplementary Fig. S1B; all \( P > 0.05 \)). These reporter assay data were concordant with levels of the endogenous Cdh1 and SnaI2 genes and respective gene products when Myc-CaP cells overexpressed Twist1 or Twist1-F191G stably (Fig. 2C and Supplementary Fig. S1C–E). Twist1-F191G bound the Cdh1 promoter as well as Twist1 wild-type in Myc-CaP cells according to ChIP-qPCR (Supplementary Fig. S1F). These results suggest that the Twist box domain is required for the full transcriptional activity of Twist1.

The Twist box mutant is partially defective for induction of EMT markers in prostate cancer cells

Metastasis is a complex series of discrete events that a neoplastic cell must traverse (36). These serial events include: loss of cell-to-cell adhesion, migration and invasion into the local extracellular matrix, intravasation into the vasculature, resistance to anoikis, extravasation into the parenchyma of distant tissues, and then colonization into a macroscopic metastatic tumor. To ascertain the role of the Twist box domain in a subset of these metastatic steps in vivo and in vitro, stable isogenic cell lines expressing Twist1 and Twist1-F191G in Myc-CaP and PC3 prostate cancer cells were established (Western blot analysis, Fig. 2C; and immunofluorescence, Fig. 2D, top row and Supplementary Fig. S2A and S2B). Consistent with an EMT marker profile, stable Twist1 overexpression led to downregulation of epithelial markers E-cadherin and ZO-1 in Myc-CaP cells and in PC3 cells and upregulation of the mesenchymal marker vimentin in both Myc-CaP and PC3 cells (Western blotting and immunofluorescence shown in Fig. 2C and D and qPCR shown in Supplementary Figs. S2A and S2B). The Twist box mutation, Twist1-F191G, resulted in a reduced ability to downregulate E-cadherin and ZO-1 and upregulate vimentin in Myc-CaP cells (Fig. 2C and D and Supplementary Figs. S2A and S2B). A similar, but less dramatic loss of an EMT marker profile was seen with PC3 cells stably overexpressing Twist1-F191G (Fig. 2C and D; Supplementary Fig. S2C and S2D show immunofluorescence quantification of PC3 cells; and Supplementary Fig. S3B and S3C show qPCR for CDH1 and VIM). The findings from these prostate cancer cell lines overexpressing Twist1 and Twist1-F191G suggest that the Twist1 box domain is required for Twist1 to induce a full EMT marker profile.

Twist1 overexpression increases cellular motility that is partially defective in the Twist1-F191G mutant

We next examined Twist1-induced cell migration and correlates of cell mechanics using the scratch/wound-healing model. For this study, we made a scratch into an ensemble of confluent Myc-CaP cells (Fig. 3A) and measured the spatiotemporal changes in forced motions of microbeads anchored to the cytoskeleton through integrin cell adhesion receptors (15, 16). Using MTC, we measured cytoskeletal stiffness (\( g^2 \)) and internal friction (\( g^3 \)) before, immediately after, and 24-hours after making a scratch. Over five decades of frequency, we found no differences in stiffness \( g^2 \) and friction \( g^3 \) between isogenic Myc-CaP cell ensembles (Twist1 vs. Vector) before or immediately after a scratch (Supplementary Fig. S4A and S4B). By 24 hours, however, Twist1-overexpressing cells infiltrated more into the site of the scratch wound than Vector control cells (Fig. 3A) and showed appreciably higher cytoskeletal stiffness (Fig. 3B and Supplementary Fig. S4C) and friction (Fig. 3C and Supplementary Fig. S4C). At 24-hour after making a scratch, Twist1-overexpressing cell ensembles exhibited a 1.6-fold higher stiffness than Vector control ensembles (Fig. 3D). Most striking was the greatest differences of cytoskeletal stiffness between Twist1 and Vector control cell ensembles were localized at the leading migratory front (Fig. 3A and E). Twist1-overexpressing cell ensembles had progressively decreasing cytoskeletal stiffness with increasing distance from the leading migratory edge (1st > 2nd > 3rd > 4th cell layers; Fig. 3A and E).

We then directly assessed the requirement of the Twist box domain for Twist1-induced cell invasive potential using the scratch/wound-healing assay in vivo. Importantly, Twist1 overexpression did not increase the proliferative potential of Myc-CaP or PC3 cells (Supplementary Fig. S5). Myc-CaP cells overexpressing Twist1 migrated 2.5-fold faster than Vector control Myc-CaP cells (Fig. 3F and G, \( P < 0.05 \)). The Twist1-F191G—overexpressing cells were significantly less migratory than Twist1—overexpressing cells, but still migrated more than the Vector (1.5-fold; Fig. 3F and G, both comparisons \( P < 0.05 \)). The same trends for cell migration were also observed in PC3 cells (Supplementary Fig. S6).

Cancer cell migration and invasion entail the ability of malignant cells to exercise contractile force upon their surroundings (37). Using traction microscopy (13, 14), we interrogated the force-generating capacity of single Myc-CaP and PC3 cells overexpressing Twist1 (Fig. 3H–J and Supplementary Fig. S7). Compared with Vector, the Twist1-overexpressing Myc-CaP and PC3 cells showed...
Increased cell spreading area and net contractile moment, a scalar measure of the cell’s contractile strength (Fig. 3I and J), $P < 0.01$ both measurements and Supplementary Fig. S7B and C, $P < 0.001$ both measurements). The Twist box mutant displayed less cell spreading and lower net contractile moment compared with Twist1 (Fig. 3I and J, $P < 0.05$ for both measurements). These single-cell biophysical data corroborated our results with bulk migration assays. Collectively, these data suggested that the Twist box domain was required for increasing cytoskeletal force generation in prostate cancer cells that was associated with the full migratory potential of Twist1.
The Twist box is required for Twist1-induced prostate cancer invasion, cell death resistance, and anchorage-independent growth in vitro

Both Myc-CaP and PC3 cells overexpressing Twist1 showed an increased invasiveness compared with Vector control cells using a Matrigel-coated Transwell invasion assay (Fig. 4A and B, both $P < 0.05$). The Twist box mutant was completely defective for invasion in Myc-CaP cells compared with Twist1 wild-type (Fig. 4A, $P < 0.05$) and trended toward being less invasive in PC3 cells (Fig. 4B, $P = 0.156$). Similar to motility, the Twist box is at least partially required for Twist1-induced prostate cancer invasion.

The resistance to anoikis facilitates metastasis of cancer cells to distant organs. Both Twist1-overexpressing Myc-CaP and PC3 cells showed decreased apoptosis when grown in suspension compared with their isogenic Vector control cells (Fig. 4C–F, both cell lines $P < 0.05$). The Twist1-F191G mutant in Myc-CaP and PC3 cells were similar to their isogenic Vector control cells (Fig. 4C–F, both cells $P > 0.47$) for anoikis resistance. We also observed that the Twist box was required to confer radioresistance.
to prostate cancer cells (Supplementary Fig. S8). Altogether, these data show that Twist1 overexpression can confer resistance to multiple cell death stimuli, and that the Twist box domain is required for these Twist1 activities in prostate cancer cells.

The in vitro anchorage-independent growth of Myc-CaP and PC3 cells stably overexpressing Twist1 and the Twist box mutant was conducted (Fig. 4G–J). Both Twist1-overexpressing Myc-CaP and PC3 cells showed increased frequency of colonies in soft agar compared with their isogenic Vector control cells (Fig. 4G–J, both cells P < 0.01). In addition, Myc-CaP cells overexpressing Twist1 had colonies of larger size (Fig. 4G). Myc-CaP and PC3 Twist box mutant cells had a similar frequency of colonies in soft agar compared with their isogenic Vector control cells (Fig. 4G–J, both cells P > 0.05). These general results were repeated and confirmed in a third prostate cancer cell line, 22Rv1, stably overexpressing Twist1 and Twist1-F191G (Supplementary Fig. S9). These data further confirm the importance of the Twist box domain for aggressive in vitro prostate cancer cell behavior induced by Twist1.

The Twist box is required for Twist1-induced prostate cancer metastasis in vivo

Using a subcutaneous tumor graft assay, we did not observe Twist1 or Twist1-F191G overexpression increasing the in vivo primary tumorigenic potential or primary tumor
Figure 6. The Twist1 global gene expression profile in Myc-CaP prostate cancer cells is greatly attenuated by a mutation in the Twist1 box domain. A, gene expression analysis of Myc-CaP cells stably expressing Vector, Twist1 (WT), and Twist1-F191G by microarray revealed a larger set of genes that were differentially expressed between Twist1-overexpressing cells and Vector control compared with the Twist1-F191G–mutant and Vector control cells. Gene expression between groups was conducted by empirical Bayesian moderated ANOVA, and genes were considered differentially expressed if B > 0 following Benjamini–Hochberg false discovery rate (FDR). B, heatmap visualization of supervised clustering analysis of gene expression from Myc-CaP cells expressing Vector, Twist1 (WT), and Twist1-F191G show that the Twist1 box mutant Twist1-F191G has a gene expression profile more similar to the Vector control cells. Each column represents a Myc-CaP microarray sample, and each row represents median-centered expression values for a single gene. High expression is indicated in dark grey, intermediate expression in black, and low expression in light grey. C, selected gene sets from the curated Molecular Signatures database that were overrepresented (P < 0.05, one-way Fisher exact test, Benjamini–Hochberg FDR) in the set of genes differentially regulated by Twist1 but not by Twist1-F191G that are relevant to phenotypic differences between prostate cancer cells overexpressing Twist1 versus Twist1-F191G as shown in this study (relative overexpression is indicated in green and relative repression by red). Twist1 but not Twist1-F191G overexpression resulted in Hoxa9/HOXA9 overexpression in (D) Myc-CaP and (E) PC3 cells (*, P < 0.05; **, P < 0.01; and ***. P < 0.001 by Mann–Whitney test). F, schematic of the promoter region of Hoxa9 with E-box containing regions (ER). Twist1 and Twist1-F191G bind to ER4 and ER5 by ChIP-qPCR. Inverted triangles are E-box sequences and arrows represent qPCR oligo sets flanking each ER. * indicates P < 0.05 as compared with Vector; & indicates P < 0.05 as compared with Twist1. G, summary of phenotypes for Twist1 and the Twist box mutant in Myc-CaP and PC3 prostate cancer cells. T, Twist1; F, Twist1-F191G mutant; and V, Vector control.

growth of Myc-CaP, PC3, or 22Rv1 cells (Supplementary Fig. S10). The metastatic potential of Twist1 and Twist1-F191G–overexpressing Myc-CaP cells was assessed using the experimental lung metastasis assay. Twist1 overexpression significantly increased the ability of Myc-CaP cells to colonize the lungs and form macroscopic metastases in vivo (Fig. 5A and B; 10/18 mice with Twist1-overexpressing Myc-CaP cells vs. 2/17 mice with isogenic Vector control cells, **P = 0.0116). The Twist box mutant overexpressing Myc-CaP cells lost some potential to form macroscopic lung metastases in vivo and had an intermediate phenotype to Vector and Twist1 Myc-CaP cells (Fig. 5B; 4/12 mice with Twist1-F191G–overexpressing Myc-CaP cells, P>0.2). The tumor cell morphology from Twist1 and Twist1-F191G–overexpressing cells was not different (Fig. 5C). Interestingly, mice injected tail vein with Twist1-overexpressing Myc-CaP cells showed extrathoracic metastases to distant subcutaneous tissues, abdominal organs, and distant lymph nodes.
(Fig. 5D–F). Cells injected in the venous circulation seed the lungs and therefore must undergo the full metastatic pathway to produce extrathoracic metastases. Twist1 overexpression significantly increased the frequency of mice with extrathoracic metastases (Fig. 5E; 11/18 mice with Twist1-overexpressing Myc-CaP cells compared with 1/17 mice with isogenic Vector control cells, P = 0.0009). The Twist box was required for Myc-CaP cells to undergo the full metastatic pathway and give rise to extrathoracic metastases (Fig. 5E; 2/12 mice injected with Twist1-F191G-overexpressing Myc-CaP cells, P = 0.0256 compared with Twist1; and P = 0.553 compared with Vector control cells). The Myc-CaP identity of lung tumors and extrathoracic metastases was confirmed by Myc IHC (Fig. 5G). These results show that the Twist box domain is required for Twist1-induced metastasis of prostate cancer cells in vivo.

Gene expression profiling reveals Hoxa9 as a direct target of Twist1 that is partially required for Twist1-induced prometastatic phenotypes

Global gene expression analysis on the isogenic Myc-CaP lines revealed several genes differentially expressed in each pairwise comparison. Compared with Vector control, Twist1 overexpression altered the expression of 424 genes. Twist1-F191G overexpression compared against Vector control altered the expression of much fewer genes, 53 genes, and the majority (28/53) of these genes were also observed with Twist1 overexpression. Between Twist1 and Twist1-F191G, 158 genes were altered, of which the majority were also altered in Twist1 (81/158; Fig. 6A and Supplementary Tables S1 and S2). This expression pattern is consistent with Twist1-F191G having a greatly attenuated transcriptional program compared with wild-type Twist1 (Fig. 6B). These global gene expression profiling data are consistent with our promoter reporter assays conducted above suggesting that the Twist box domain is required for the full transcriptional activity of Twist1 (Fig. 2B and Supplementary Fig. S1). These findings are highly suggestive of a transcriptional program for the phenotypic differences observed between prostate cancer cells overexpressing Twist1 and those overexpressing the Twist box mutant.

GSEA (38) was used to identify gene sets which were overrepresented in Twist1 but not Twist1-F191G. Many of the overrepresented gene sets were related to phenotypes which we directly assayed, aggressive cellular behavior and metastasis, and were observed with overexpression of Twist1 but not Twist1-F191G (Fig. 6C). One gene set of interest was directed by the homeobox transcription factor, Hoxa9, which is strongly implicated in leukemogenesis. Furthermore, the Hox homolog HOXA9 had been shown previously to interact physically with Twist and antagonize repression of p53 to genotoxic stressors. Thus, we confirmed our microarray data by qPCR and Western blotting showing that Twist1 overexpression resulted in Hoxa9/HOXA9 overexpression in Myc-CaP and PC3 (Fig. 6D and E; both P < 0.01 by qPCR and Supplementary Fig. S1A and S1B by Western blotting). Twist1 also bound to the Hoxa9 promoter in a region containing canonical E-box sequences as shown by ChIP-qPCR (Fig. 6F). Consistent with our global gene expression data, the Twist box mutant was unable to upregulate the expression of Hoxa9/HOXA9 overexpression in Myc-CaP and PC3 and was similar to Vector control (Fig. 6D and E; both P < 0.05 by qPCR and Supplementary Fig. S1A and S1B by Western blotting). However, the Twist1-F191G mutant was still capable of binding to the Hoxa9 promoter by ChIP-qPCR, suggesting that the Twist box was required for the full transcriptional activity of Twist1 (Fig. 6F and summarized differences between in vitro and in vivo phenotypes of Twist1 and Twist1-F191G in Fig. 6G).

Interestingly, we found that many of the in vitro prometastatic phenotypes of Twist1 overexpression in Myc-CaP cells were significantly blunted following short hairpin RNA (shRNA)-mediated knockdown of Hoxa9 (Fig. 7). Three separate shRNA constructs against Hoxa9 were each able to knockdown Hoxa9 mRNA and protein expression in Myc-CaP cells overexpressing Twist1 (Fig. 7A; P < 0.05 for qPCR). Hoxa9 knockdown in these Twist1-overexpressing cells resulted in a reduction in Twist1-induced cellular migration, invasion, anoikis resistance, and soft agar clonogenicity (Fig. 7B–G; P < 0.05 all at least). Collectively, these data suggest that Twist1 imparts prometastatic phenotypes on prostate cancer cells, in part, by directly upregulating Hoxa9 expression.

Discussion

Our study shows that Twist1 overexpression in prostate cancer cells induces an EMT phenotype, augments migration, invasion, and resistance to anoikis and metastasis. We show that the highly conserved Twist box domain is required for many of these properties of Twist1 associated with aggressive tumor cell behavior in vitro and most importantly for metastasis in vivo. We also show that the Twist box domain is required for the full transcriptional activity of Twist1 and facilitates these prometastatic cellular functions by directing specific transcriptional programs. We show that Twist1 directly regulates the transcriptional prometastatic target, Hoxa9, which is at least partially required for Twist1-induced prometastatic phenotypes in prostate cancer cells.

The Twist box is highly conserved among vertebrates and is critical for the role of Twist1 in development as shown by inactivating mutations in this region of the human gene resulting in the Saethre–Chotzen syndrome, characterized primarily by craniosynostosis (5, 39). Consistent with humans, the Charlie Chaplin mouse strain with craniosynostosis and hind-limb abnormalities results from a S192P substitution mutation in the Twist box domain (40). Mechanistically, Twist1 binds to the Runx2 transcription factor via the Twist box and inhibits the Runx2 transcriptional program necessary for osteoblast differentiation. Similarly, Twist1 binds Sox9 via the Twist box and inhibits Sox9-dependent transcriptional programs required for chondrocyte differentiation (41). Twist1 may also directly modulate transcription of target genes, and the Twist box was shown to be both necessary and sufficient for this transactivation activity (34). The Twist box transactivation domain likely
adopts an α-helical structure, and the three amino acids, Leu-187, Phe-191, Arg-195, are essential for transactivation function and may occupy the same three-dimensional surface of this α-helix. Twist1 has been shown to directly upregulate the expression of several target genes important for cancer progression like Akt2 expression, which enhances cell migration, invasion, and resistance to chemotherapy (42). In agreement with these findings in breast cancer, our expression profiling of Twist1-overexpressing prostate cancer cells were similar to gene signatures consistent with increased cell migration, invasion, and resistance to apoptosis. However, whether the Twist box mediates a transcriptional program required for Twist1-induced metastasis in prostate cancer cells by actively inhibiting another transcription factor or by directly regulating downstream target genes requires further study.

The role of the Twist box in cancer-related functions has only recently been appreciated. In a recent study, the Twist box was required for Twist1 binding to the NF-κB subunit RELA to activate transcriptional activity, increased DNA-binding affinity to the interleukin 8 (IL-8) promoter and transcriptional activation that was required for breast cancer cell invasion in vitro (43). We did not observe direct Twist box-dependent regulation of IL-8 by Twist1 in our system, but in agreement with this study, we did observe Twist box-dependent gene sets consistent with NF-kB-regulated inflammatory genes. The Twist box has also recently been shown to bind p53 and induce destabilization via MDM2-
mediated degradation in sarcoma cells (44). This mechanism is separate from Twist1 indirect regulation of p53 by modulating the ARF/MDM2/p53 pathway (45). We do not believe that Twist box-dependent p53 regulation explains the Twist1-induced metastatic phenotypes we observed in prostate cancer cells as PC3 cells are TP53-null and a similar Twist1-F191L substitution mutation in the Twist box did not affect Twist1-p53 interaction (44). Our study findings require validation in other cancer histologies before our results can be generalized further and confirmation using autochthonous transgenic models of tumorigenesis and spontaneous metastasis models is needed. Despite these limitations, our current study confirms the recent in vitro data showing the importance of the Twist box domain for the protumorigenic activities of Twist1. Importantly, our study shows for the first time the requirement of the Twist box for the prometastatic functions of Twist1 in vitro and in vivo.

TWIST1 expression levels seem to be correlated with prostate cancer aggressiveness and factors associated with lethal metastatic disease (Fig. 1; refs. 7, 33). The downregulation of Twist1 in androgen-independent prostate cancer cells increased their sensitivity to anticancer drugs and suppressed their migration and invasion abilities, suggesting Twist1 inactivation as a potential therapeutic strategy (7). TWIST1 expression during postnatal life is restricted tightly to a subpopulation of mesoderm-derived tissues, and limited studies suggest that Twist1 inhibition systemically may be well tolerated (46). Furthermore, our previous study suggested that suppression of Twist1 to physiologic levels in vivo is sufficient for anticancer effects (11). However, the direct inhibition of Twist1 as a therapeutic maneuver still poses a few potential challenges. First, Twist1 is a pleiotropic transcription factor essential for mammalian development (47). Second, bHLH transcription factors have been difficult to target directly with small molecules (48). A solution to these issues is dissecting what are the critical domains of Twist1 and what are the crucial Twist1 downstream transcriptional targets that are required for Twist1-dependent tumorigenicity and prometastatic functions. Using comparative gene expression profiling of Twist1 and Twist box mutant cells, we discovered Hoxa9 as a novel direct gene target of Twist1 that was required, in part, for many Twist1-induced prostate cancer prometastatic phenotypes in vitro. Although there is a rich literature on the oncogenic role of Hoxa9 in leukemia (49), only one recent report has suggested a role for Hoxa9 in prostate cancer (50). Thus, we have uncovered a novel mechanism involving Twist1 and Hoxa9 oncoproteins collaborating to facilitate prostate cancer progression and metastatic cellular behavior.

In conclusion, these data herein have increased our insight into the structure–function relationships of the Twist1 oncoprotein in cancer and point to the Twist box as a critical domain required for directing transcriptional prometastatic programs in prostate cancer cells. Our findings suggest therapeutic measures against TWIST1-overexpressing prostate cancer cells should be minimally directed against the Twist box domain and Twist1-regulated transcriptional targets such as Hoxa9.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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