Supplementary Information

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

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Supplementary Materials and Methods

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Supplementary Materials and Methods

Microarray data acquisition and analysis

Microarrays were performed using GeneChip WT cDNA Synthesis and amplification Kit and WT terminal labeling Kit (Affymetrix, Santa Clara, CA). The labeled ssDNA was hybridized to the GeneChip Mouse Gene 1.0 ST array (Affymetrix, Santa Clara, CA), washed with the Fluidics station 450 and array scanning was performed (1). Arrays were normalized using the Robust Multichip Average (RMA) (2) in the oligo Bioconductor package at the transcript level. Microarrays of this study passed quality control metrics. Data analysis was performed in R using the LIMMA package. Expression was modeled with a linear model accounting for individual transcription factors and mean expression of transcripts annotated to *TWIST1*, which was found to correlate with global gene expression changes. Genes with Benjamini-Hochberg (3) *p*-values below 0.05 were considered statistically significant. GSEA was performed using the C2 Curated Gene Sets collection from the Molecular Signature Database 3.0 and statistical comparisons by Fisher's Exact Test based upon these significant genes. Gene sets with Benjamini-Hochberg FDR adjusted $p < 0.05$ were considered overrepresented. The R code used for this analysis is below

```
library(oligo)
library('ClassDiscovery')
library('AnnotationDbi')
library('limma')
library('pd.mogene.1.0.st.v1')
library('AnnotationDbi')
library('gplots')
library('KEGG.db')
library('org.Mm.eg.db')
library('GO.db')
library('annotate')
library('mogene10sttranscriptcluster.db')
library('mogene10stprobeset.db')
library('GSA')
library('GSEABase')
```

```
celFiles <- list.files(path=
'G:/Project - PCa Twist1 Mutant Gene Expression/Raw CEL Files',
    pattern='cel',ignore.case=T,full.names=T)
rawExpressionData <- read.celfiles(celfile.path=celFiles)
expressionData <- rma(rawExpressionData,target='core')
genes2Probes <- revmap(as.list(mogene10sttranscriptclusterSYMBOL))
expressionData <- exprs(expressionData)[unique(unlist(genes2Probes)),]
Annotation <- cbind(seq(from=1,to=15,by=1),
                    rep(c('VEC','WT','AQA','DQD','F191G'),3),
                    rep(seq(1,3),each=5),
                    c('JF_A1_1_(MoGene-1_0-st-v1).CEL',
                      'JF_A2_2_(MoGene-1_0-st-v1).CEL',
                      'JF_A3_3_(MoGene-1_0-st-v1).CEL',
                      'JF_A4_04_(MoGene-1_0-st-v1).CEL',
                      'JF_A5_5_(MoGene-1_0-st-v1).CEL',
                      'JF_A6_6_(MoGene-1_0-st-v1).CEL',
                      'JF_A7_7_(MoGene-1_0-st-v1).CEL',
                      'JF_A8_08_(MoGene-1_0-st-v1).CEL',
                      'JF_B1_9_(MoGene-1_0-st-v1).CEL',
                      'JF_B2_10_(MoGene-1_0-st-v1).CEL',
                      'JF_B3_11_(MoGene-1_0-st-v1).CEL',
                      'JF_B4_12_(MoGene-1_0-st-v1).CEL',
                      'JF_B5_13_(MoGene-1_0-st-v1).CEL',
                      'JF_B6_14_(MoGene-1_0-st-v1).CEL',
                      'JF_B7_15_(MoGene-1_0-st-v1).CEL'),
                    c(rep('A',8),rep('B',7)))

colnames(Annotation) <- c('sample','knock-in','replicate','CelFile','batch')
row.names(Annotation) <- Annotation[,'CelFile']

#create vectors id'ing samples by batch and by knockin
batch.trts <- factor(Annotation[colnames((expressionData)),'batch'])
exp.trts <- factor(Annotation[colnames((expressionData)),'knock-in'])

#pull the filenames of the vector samples
VECSamples <-
colnames(expressionData)[grep('VEC',Annotation[colnames(expressionData),'knoc
k-in'])]

#initialize a vector to track Twist expression
twst.trts <- rep(0, ncol(expressionData))
names(twst.trts) <- colnames(expressionData)

expressionProbeData <- rma(rawExpressionData,target='probeset')
genes2Probesset <- revmap(as.list(mogene10stprobesetSYMBOL))

#pull the Twist expression levels
twistValues <- exprs(expressionProbeData)[genes2Probesset[['Twist1']],]
twistValues <- twistValues[which.max(apply(twistValues,1,function(x){max(x)-
min(x)})),]
#Sets the value of the Twist status vector for the VEC knock-ins equal to the
mean-centered

#twist expression of the samples
twst.trts[VECSamples] <- twistValues[VECSamples] -
mean(twistValues[VECSamples])
#sets the value of the non-VEC samples to be centered to the mean
#twist expression for the remaining samples
twst.trts[setdiff(colnames(expressionData),VECSamples)] <-
twistValues[setdiff(colnames(expressionData),
   VECSamples)] - mean(twistValues[setdiff(colnames(expressionData),
    VECSamples)])
```

```
#sets the model matrix for the batch correction by combining the knockin,
batch, and twist expression factors
batchDesign <- model.matrix(~0+exp.trts+twst.trts+batch.trts)
batchFit <- lmFit(expressionData, batchDesign)
#subtract out batch and twist expression effects
batchCorrected <- expressionData -

batchFit$coefficients[,c('batch.trtsB','twst.trts')]%*%t(batchDesign[,c('batc
h.trtsB','twst.trts')])
#set up contrast matrix for WT v VEC
compSample <- which(Annotation[colnames((expressionData)),'knock-
in']%in%c('VEC','WT','F191G'))

exprsRange <- apply(batchCorrected[,compSample],1,function(x){max(x)-min(x)})
probesSelect <- sapply(genes2Probes,function(x){x[which.max(exprsRange[x])]})
WTVec.contrasts <- makeContrasts(exp.trtsWT-exp.trtsVEC,
                                 levels=batchDesign)
#get est. coeefs and stderrs, smoothed with bayesian technique
WTVec.contrast.fit <- eBayes(contrasts.fit(batchFit[probesSelect,],
                                           WTVec.contrasts))
WTVec.contrast.fit$genes$Symbol <-
getSYMBOL(WTVec.contrast.fit$genes$ID,'mogene10sttranscriptcluster.db')

#returns the top genes, benjamini-hochberg corrected
WTResults <-
topTable(WTVec.contrast.fit,number=length(which(topTable(WTVec.contrast.fit,a
djust.method='BH',

number=length(probesSelect))$B>0)),
                      adjust.method='BH')

#repeat process with F191G v WT
F191GWT.contrasts <- makeContrasts(exp.trtsF191G-exp.trtsWT,
                                   levels=batchDesign)
F191GWT.contrast.fit <- eBayes(contrasts.fit(batchFit[probesSelect,],
                                             F191GWT.contrasts))
F191GWT.contrast.fit$genes$Symbol <-
getSYMBOL(F191GWT.contrast.fit$genes$ID,'mogene10sttranscriptcluster.db')

F191GWTResults <-
topTable(F191GWT.contrast.fit,number=length(which(topTable(F191GWT.contrast.f
it,adjust.method='BH',

number=length(probesSelect))$B>0)),
                            adjust.method='BH')
#repeat method for F191G v Vec
F191GVec.contrasts <- makeContrasts(exp.trtsF191G-exp.trtsVEC,
                                    levels=batchDesign)
F191GVec.contrast.fit <- eBayes(contrasts.fit(batchFit[probesSelect,],
                                              F191GVec.contrasts))
F191GVec.contrast.fit$genes$Symbol <-
getSYMBOL(F191GVec.contrast.fit$genes$ID,'mogene10sttranscriptcluster.db')

F191GResults <-
topTable(F191GVec.contrast.fit,number=length(which(topTable(F191GVec.contrast
.fit,adjust.method='BH',

number=length(probesSelect))$B>0)),
                           adjust.method='BH')
#Perform analysis of overlaps
e1 <- new.env()
e1$F191GvWT <- F191GWTResults$Symbol
e1$F191GvVec <- F191GResults$Symbol
```

```
e1$WTvVec <- WTResults$Symbol
AllComps <- as.list(e1)
counts <- venn(AllComps)

#get list of WT-Vec-Only genes
WTVecOnly <- setdiff(WTResults$Symbol, union(F191GWTResults$Symbol,
F191GResults$Symbol))
WT_not_F191G <- setdiff(WTResults$Symbol, intersect(F191GResults$Symbol,
WTResults$Symbol))

#make heat map
pick_samples <- function(mutant){
    compSamples <- which(Annotation[colnames((expressionData)),'knock-
in']%in%c('VEC','WT',mutant))
    return(compSamples)
}

gene_lookup <- function (gene, samples) {
    probes <- genes2Probes[[gene]]
    data <- batchCorrected[probes[1], samples]
    return(data)
}

make_gene_table <- function(filedata, samples){
    names_list <- as.vector(filedata)
    genedata <- sapply(names_list, gene_lookup, samples)
    return(genedata)
}

samples <- pick_samples("F191G")
gene_table <- make_gene_table(WT_not_F191G, samples)
gene_matrix <-  t(data.matrix(gene_table))
colnames(gene_matrix) <- paste(Annotation[colnames(gene_matrix),'knock-in'],
                               Annotation[colnames(gene_matrix),'replicate'],
                               sep=".")
hm_lmat <- matrix(c(0,0,0,0,0,0,1, 1, 1, 2, 0, 0, 4, 0, 3), 3, 5, byrow =
TRUE)

hm_lwid <- c(1, 6, 8, 6, 1)
hm_lhei <- c(1, 12, 3)

heatmap.2(gene_matrix,
          col = redgreen (75),
          scale = "row",
          trace = "none",
          keysize = 0.5,
          margins = c(5,0),
          density.info = "none",
          dendrogram = "column",
          labRow = "",
          #lmat = hm_lmat,
          #lhei = hm_lhei,
          #lwid = hm_lwid
          )

#GSEA/GO Analysis
GSEASets <- GSA.read.gmt('c2.all.v3.0.symbols.gmt')
WTVecOnly_probes <- probesSelect[WTVecOnly$Symbol]
AllWT_probes <- probesSelect[WTResults$Symbol]
F191G_and_WT_probes <- probesSelect[intersect(WTResults$Symbol,
F191GResults$Symbol)]
WT_not_F191G_probes <- probesSelect[setdiff(WTResults$Symbol,
intersect(WTResults$Symbol, F191GResults$Symbol))]
```

```
names(WTVecOnly_probes) <- toupper(names(WTVecOnly_probes))
names(AllWT_probes) <- toupper(names(AllWT_probes))
names(F191G_and_WT_probes) <-toupper(names(F191G_and_WT_probes))
names(WT_not_F191G_probes) <- toupper(names(WT_not_F191G_probes))

subsets_to_test <- list(WTVecOnly_probes, AllWT_probes, F191G_and_WT_probes,
                        WT_not_F191G_probes)

gsea_fisher <- function(sig_genes, geneset){
    geneset <- unlist(geneset)
    sig_in_set <- length(intersect(sig_genes, geneset))
    sig_not_in_set <- length(sig_genes) - sig_in_set
    not_sig_in_set <- length(geneset) - sig_in_set
    not_sig_not_in_set <- length(batchCorrected[,1]) - not_sig_in_set
    data <- matrix(c(sig_in_set, not_sig_in_set, sig_not_in_set,
not_sig_not_in_set), nrow = 2)
    test <- fisher.test(data,alternative='greater')
    return(test$p.value)
}

subset_gsea_test <- function(sig_genes){
    gsea_tests <- p.adjust(sapply(GSEASets$genesets,
function(x){gsea_fisher(names(sig_genes), x)}), method = "BH")
    names(gsea_tests) <- GSEASets$geneset.names
    sig_genesets <-gsea_tests[which(gsea_tests < .05)]
    return(sig_genesets)
}

gsea_results <- sapply(subsets_to_test, subset_gsea_test)
gsea_overlap <- venn(list(names(gsea_results[[1]]),
                          names(gsea_results[[2]]),
                          names(gsea_results[[3]]),
                          names(gsea_results[[4]])))
```

Chromatin Immunoprecipitation – Quantitative PCR (ChIP-qPCR)

Chromatin immunoprecipitation was performed using a SimpleChIP Enzymatic IP Kit (Cell Signaling Technology, Danvers, MA) according to the manufacturer's instructions. Briefly, cells were fixed and crosslinked by addition of 1% formaldehyde for 15 minutes. Cells were then lysed and DNA was fragmented by incubation with micrococcal nuclease for 20 minutes at 37°C.

Lysates were then immunoprecipitated by addition of 5 µg anti-Twist1 antibody (Vesuna et. al. 2008), with isotype-matched IgG as a negative control and rabbit anti-Histone H3 antibody as a positive control. Precipitations proceeded overnight at 4°C and linked chromatin was pelleted using Protein G magnetic beads. Precipitated DNA was then washed and eluted.

The concentration of target sequences containing E-boxes in the promoter regions of Hoxa9 and Cdh1 was determined by qPCR using iTaq Universal SybrGreen Master Mix (BioRad, Hercules, CA) according to the manufacturer's instructions. Primer sequences are shown in Supplemental Table 1. Concentrations were normalized to the concentration of the sequences in the input lysates.

REFERENCES

1. Zhou G, Wang S, Wang Z, Zhu X, Shu G, Liao W, et al. Global comparison of gene expression profiles between intramuscular and subcutaneous adipocytes of neonatal landrace pig using microarray. Meat Sci. 2010 Oct;86(2):440-50.

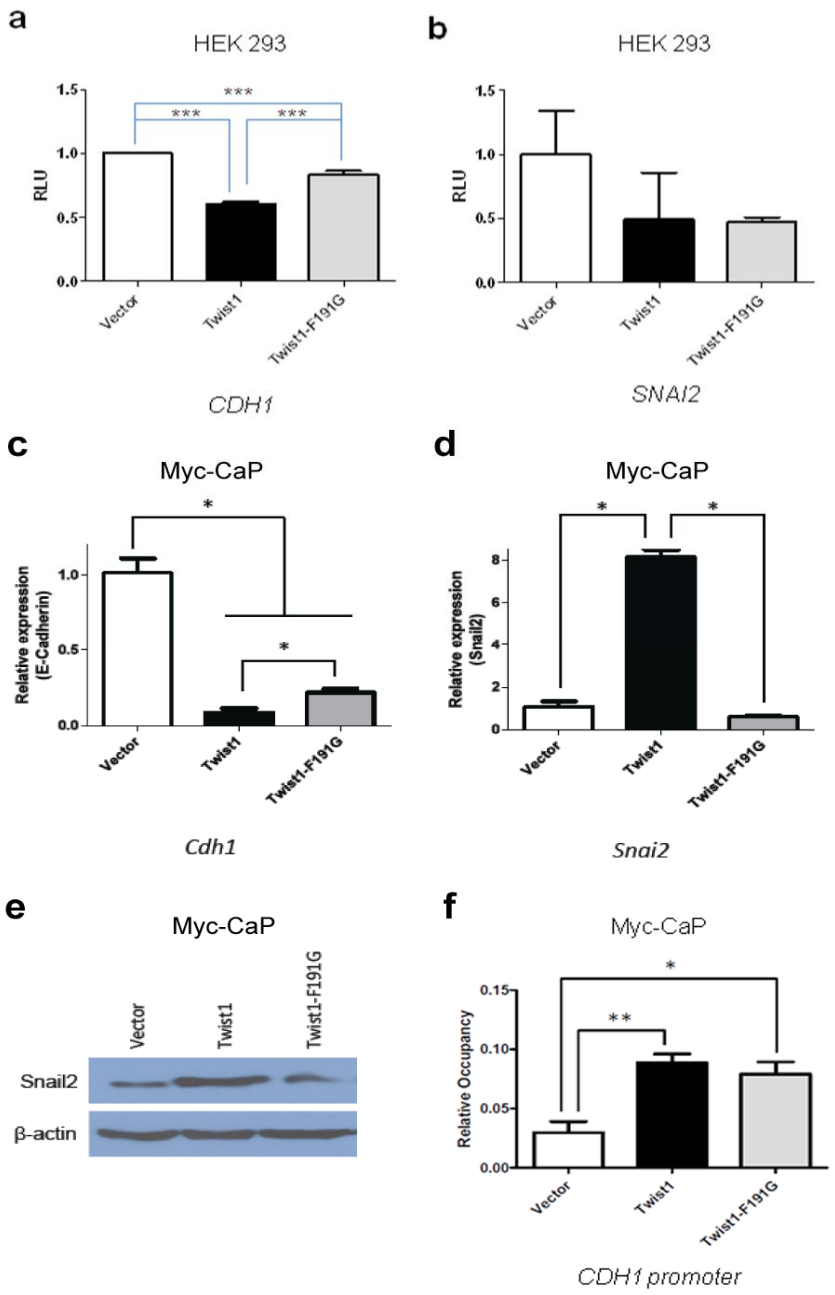
2. Carvalho BS, Irizarry RA. A framework for oligonucleotide microarray preprocessing. Bioinformatics. 2010 Oct;26(19):2363-67.

3. Benjamini Y, Hochberg Y. Controlling the false discovery rate: a practical and powerful approach to multiple testing. J. R. Stat. Soc. Ser. B-Stat. Methodol. 1995;57(1): 289–300.

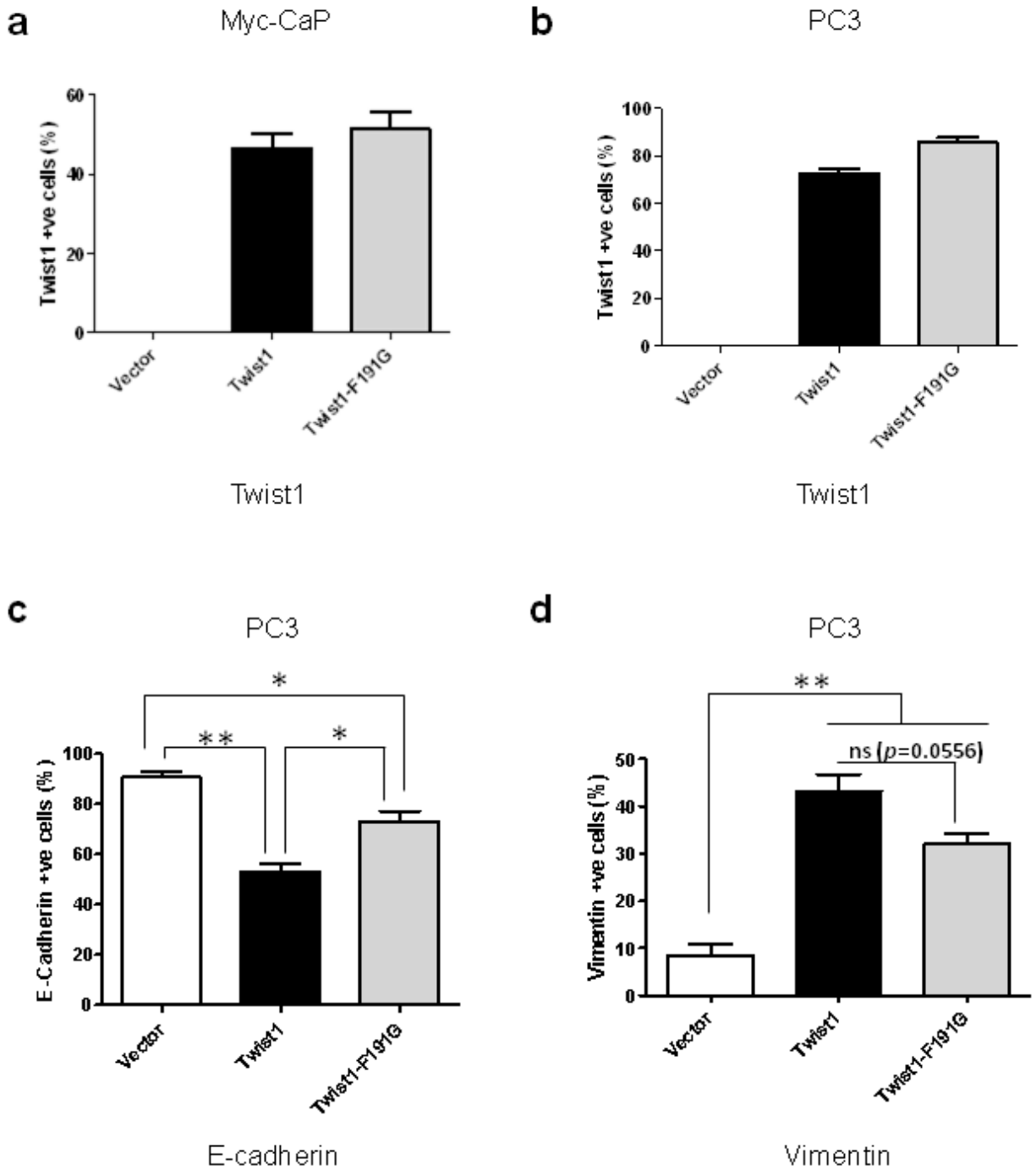
4. Casas E, Kim J, Benedesky A, Ohno-Machado L, Wolfe CJ, and Yang J. Snail2 is an Essential Mediator of Twist1-Induced Epithelial Mesenchymal Transition and Metastasis. Cancer Res. 2011 Jan; 71(1):245-254.

	Forward	Reverse	Reference
Hoxa9 promoter E-Box region 1	AACATACTCTTGGAAGGGCAC	GGGAGGAACACTGGSCATTG	This study
Hoxa9 promoter E-Box region 2	CATTTTCTGCATCCGGGTAC	GGTACTGAGTATTAAGCAGC	This study
Hoxa9 promoter E-Box region 3	GTCATTTTCCTGCAAGGCTG	CAGGGGCAACTGAGAGTAGAG	This study
Hoxa9 promoter E-Box region 4	CGAGAGCACACTGCCTTC	CCGCTAACCTGACTGTACCTGC	This study
Hoxa9 promoter E-Box region 5	GTGGCTGTCTTGGACCAGTG	CAGGAAACGCGTGGCCTAG	This study
Hoxa9 promoter E-Box region 6	CCATATCCCTGCTGTTAAC	AGGGAGAATTGTGGTGTGC	This study
Cdh1 promoter	TAGGAAGCTGGGAAG	TGCGGTCGGGCAGGG	Sideridou M, Zakopoulou R, Evangelou K, Liontos M, Kotsinas A, Rampakakis E, et al. (2011). Cdc6 expression represses E-cadherin transcription and activates adjacent replication origins. J. Cell Biol. 195:1123–40.

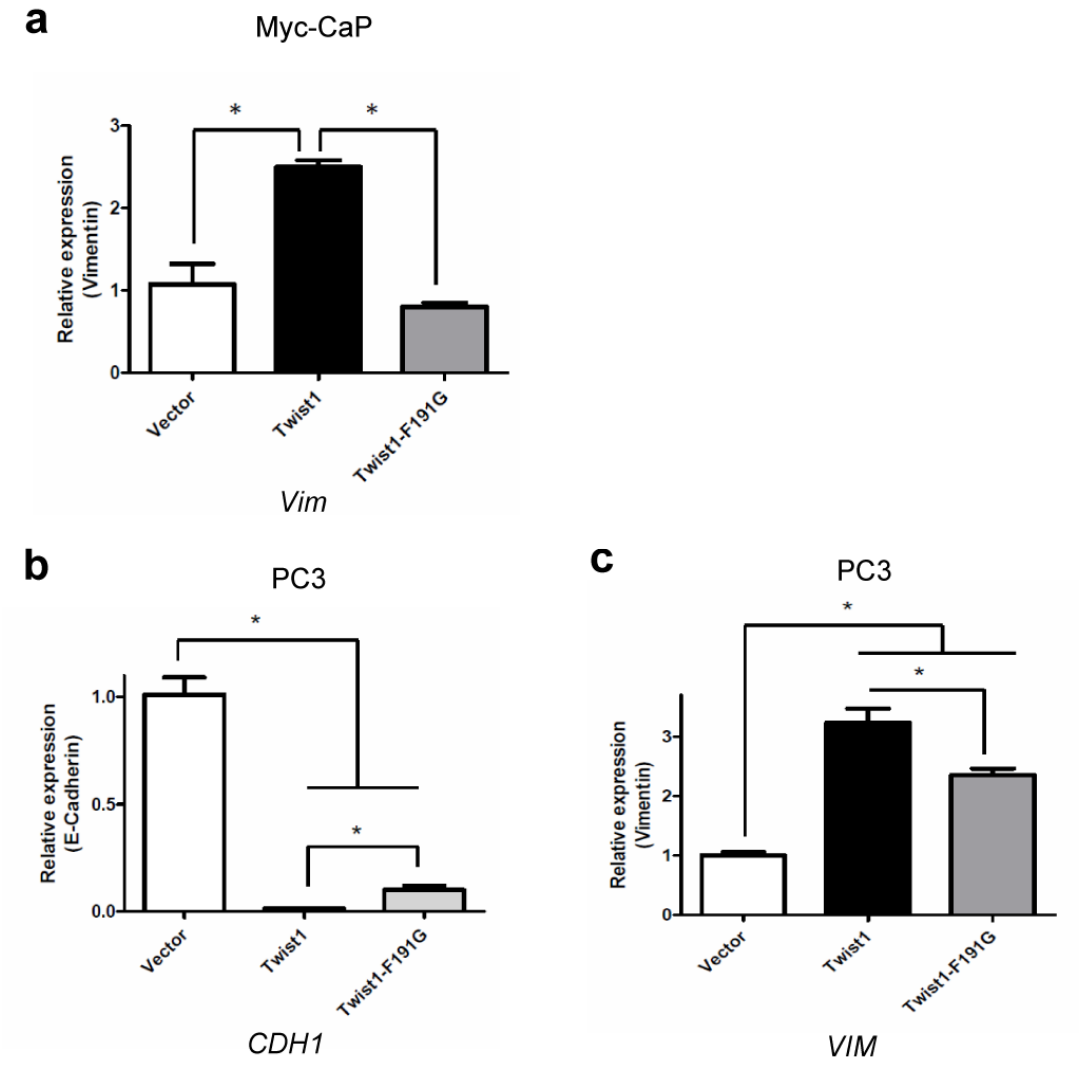
Supplementary Table S3. ChIP-qPCR oligos used in this study.



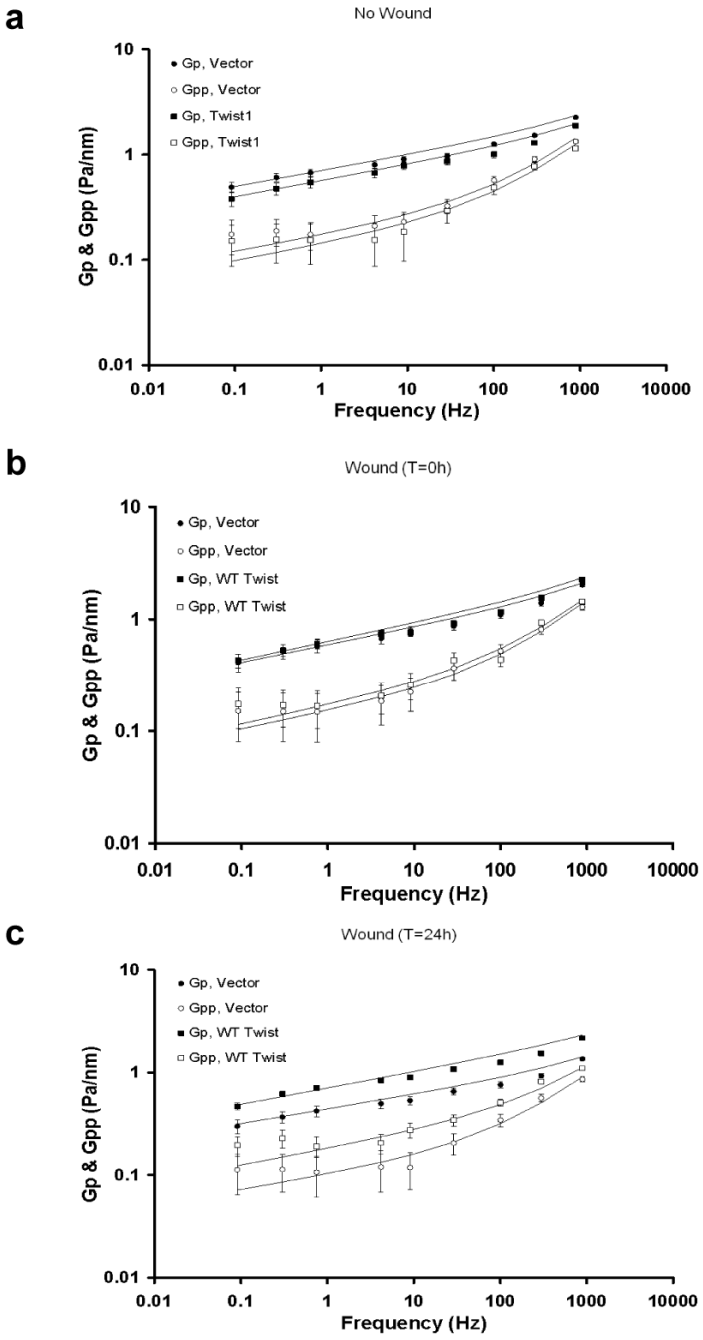
Supplementary Figure S1. The Twist box domain is required for full Twist1 transcriptional activity. Luciferase reporter assay for the (**a**) *CDH1* promoter or (**b**) *SNAI2* promoter (as in ref. 4) in HEK 293 cells transiently co-transfected with 500 ng of Twist1 or Twist1-F191G expression constructs (see Materials and Methods). The Twist box domain is necessary for Twist1 to fully repress the *CDH1* promoter *in vitro* (Mann Whitney test: ***, p <0.001). Endogenous levels of (c) *Cdh1* and (d) *Snai2* where examined by qPCR in Myc-CaP cells stably overexpressing Twist1 or Twist1-F191G and are concordant with results from promoter reporter assays (Figure 2b). Levels of (e) Snai2 by Western blotting of Myc-CaP cells were also concordant with the gene expression level data. (**f**) The Twist box mutation does not effect binding of Twist1 to the E-boxes in the *Cdh1* promoter in Myc-CaP cells as demonstrated by ChIP promoter occupancy (see Supplementary Materials and Methods; Mann Whitney test: **, p <0.01; *, p <0.05). Bars represent column mean; error bars ± SEM. Each bar represents values from three independent experiments performed in triplicate.



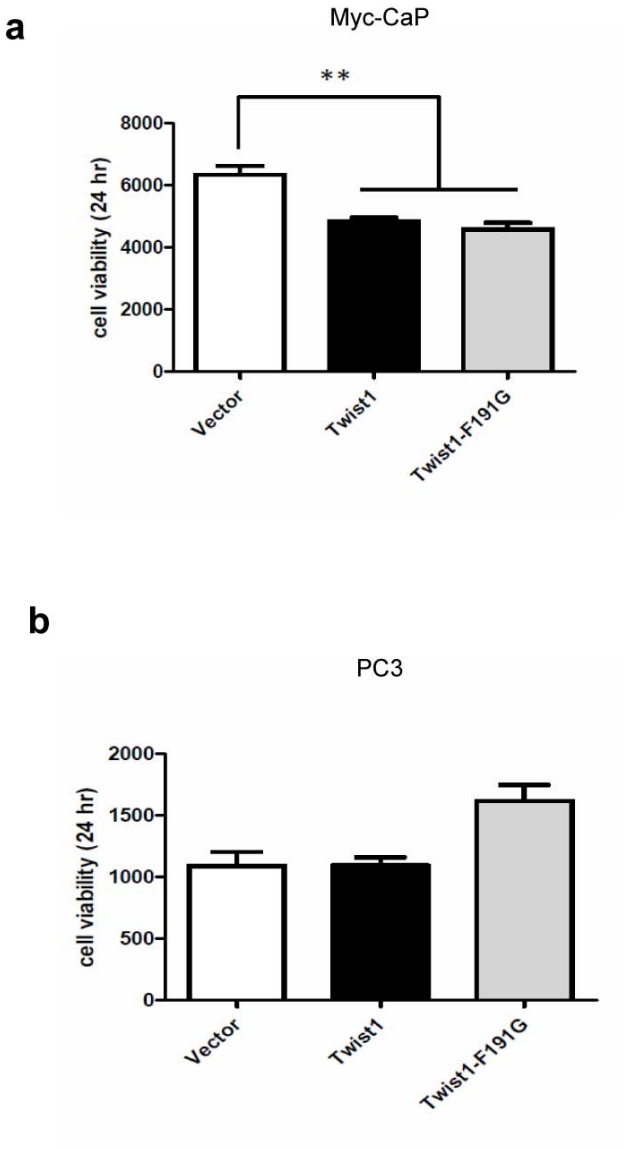
Supplementary Figure S2. Quantification of immunofluorescence from isogenic prostate cancer cell lines stably expressing Twist1 and Twist1-F191G. Twist1 immunofluorescence was performed on (**a**) Myc-CaP and (**b**) PC3 isogenic cells and quantified. (**c**) E-cadherin and (**d**) vimentin immunofluorescence was performed on PC3 isogenic cells and quantified. Five random fields at 400X magnification were scored and the percent of positive cells to the total number of cells is plotted. Bars represent column mean; error bars ± SEM.



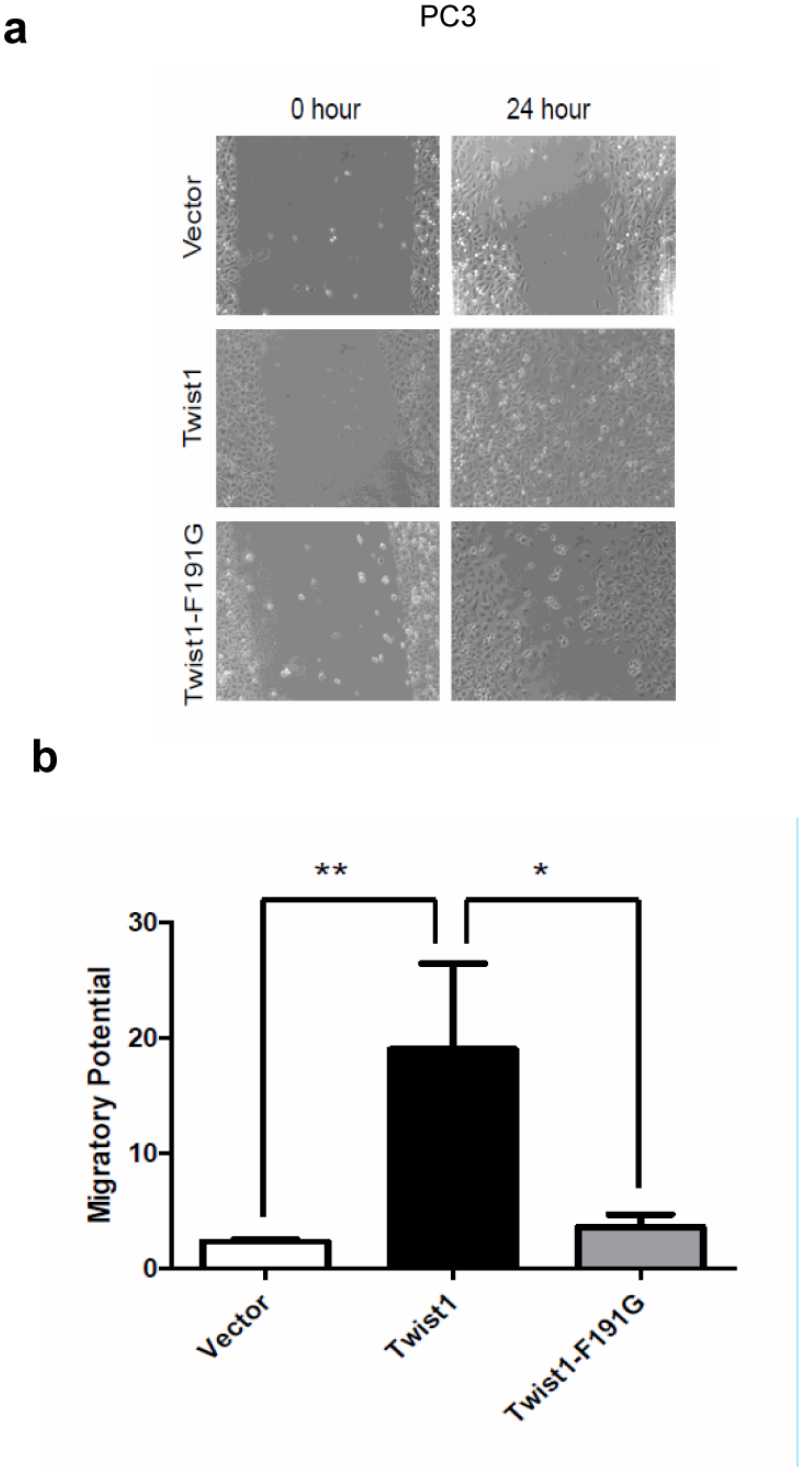
Supplementary Figure S3. The Twist box domain is required for full Twist1-induced EMT marker phenotypes of prostate cancer cells. Prostate cancer cells stably overexpressing Vector, *Twist1* or *Twist1-F191G* had qPCR performed on them for (**a**) *Vim* in Myc-CaP cells and on isogenic PC3 cells for (**b**) *CDH1* and (**c**) *VIM*. Bars represent column mean; error bars ± SEM (Mann Whitney test: *, $p < 0.05$).



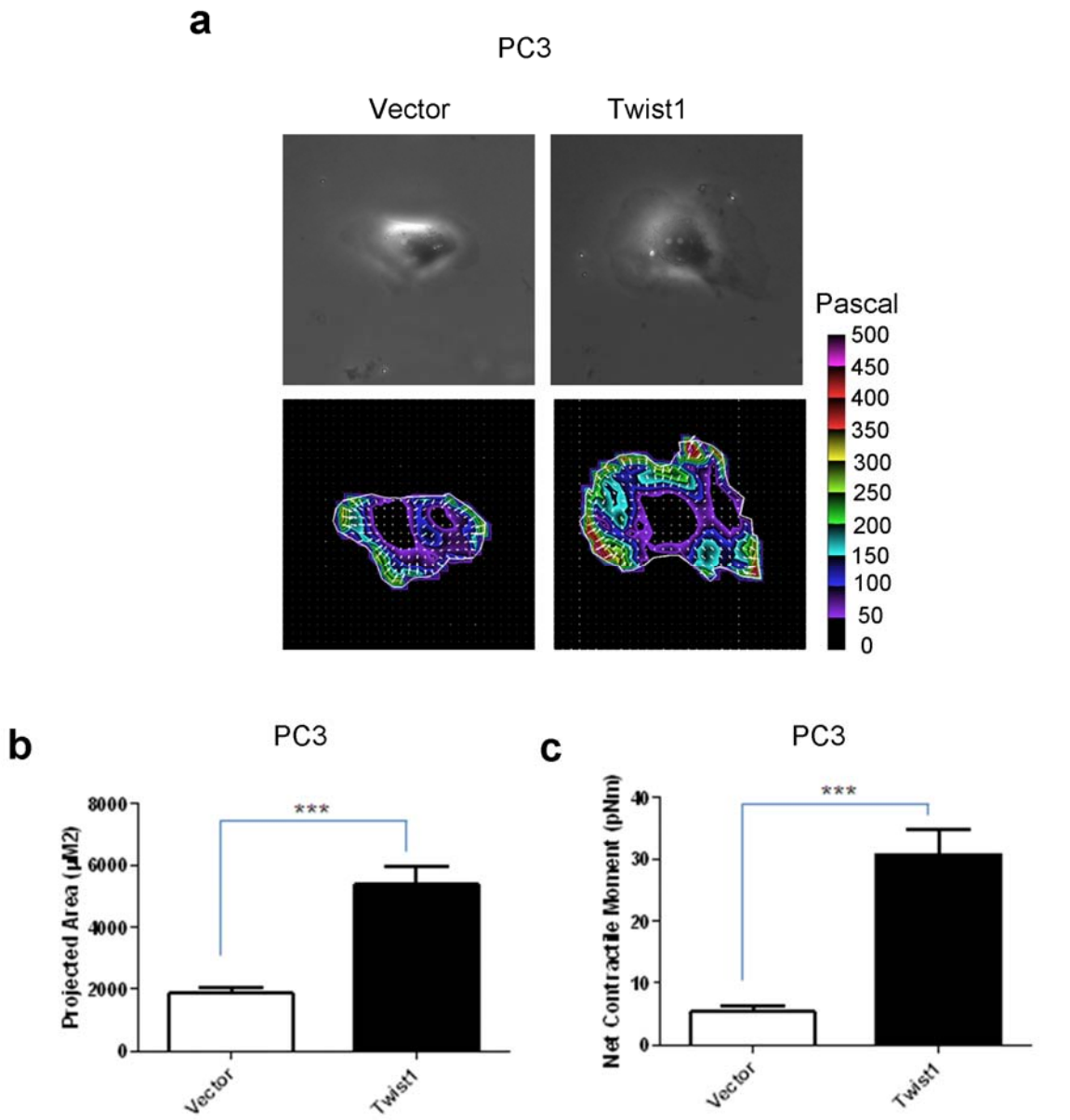
Supplementary Figure S4. Twist1 overexpression induces temporal changes in the material properties of prostate cancer cells during their migration in a wound healing assay. Both Vector control and Twist1 expressing Myc-CaP cells were plated at 150,000 cells/cm^2 on plastic wells (96-well Removawell, Immulon II: Dynetech) previously coated with type I collagen. Once cells became confluent, a line wound was made with a 200 µl pipette tip and maintained at 37^{o}C in humidified air containing 5 % CO_2. After indicated time [**a,** no wound; **b**,immediately after (T=0 hr) and (**c**) 24-hr after a scratch wound (T=24 hr)], ferrimagnetic microbeads were functionalized to the living cytoskeleton (CSK) and, using magnetic twisting cytometry (MTC), stiffness g' and loss modulus g" were measured over a physiological range of frequency (*f*). Data are presented as Geometric Mean $\pm$ SE [Vector: no wound (n= 248), T=0 hr (n=169), T=24 hr (n=337); Twist1: no wound (n= 221), T=0 hr (n=240), T=24 hr (n=396)].



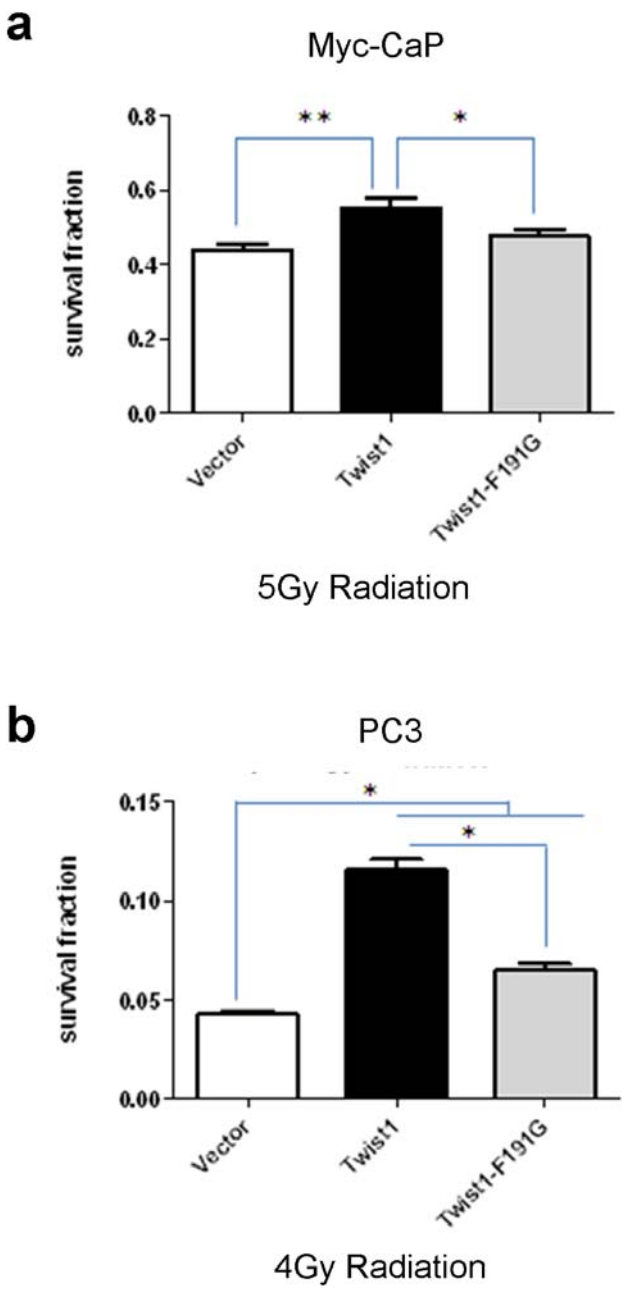
Supplementary Figure S5. The overexpression of *Twist1* or *Twist1-F191G* does not increase cellular proliferation of prostate cancer cells *in vitro*. Prostate cancer cells stably overexpressing Vector, *Twist1* or *Twist-F191G* had MTT assays performed on them and the 24-hour time point is shown. (**a**) isogenic Myc-CaP cells and (**b**) isogenic PC3 cells. Bars represent column mean; error bars ± SEM (Mann Whitney test: **, $p < 0.01$).



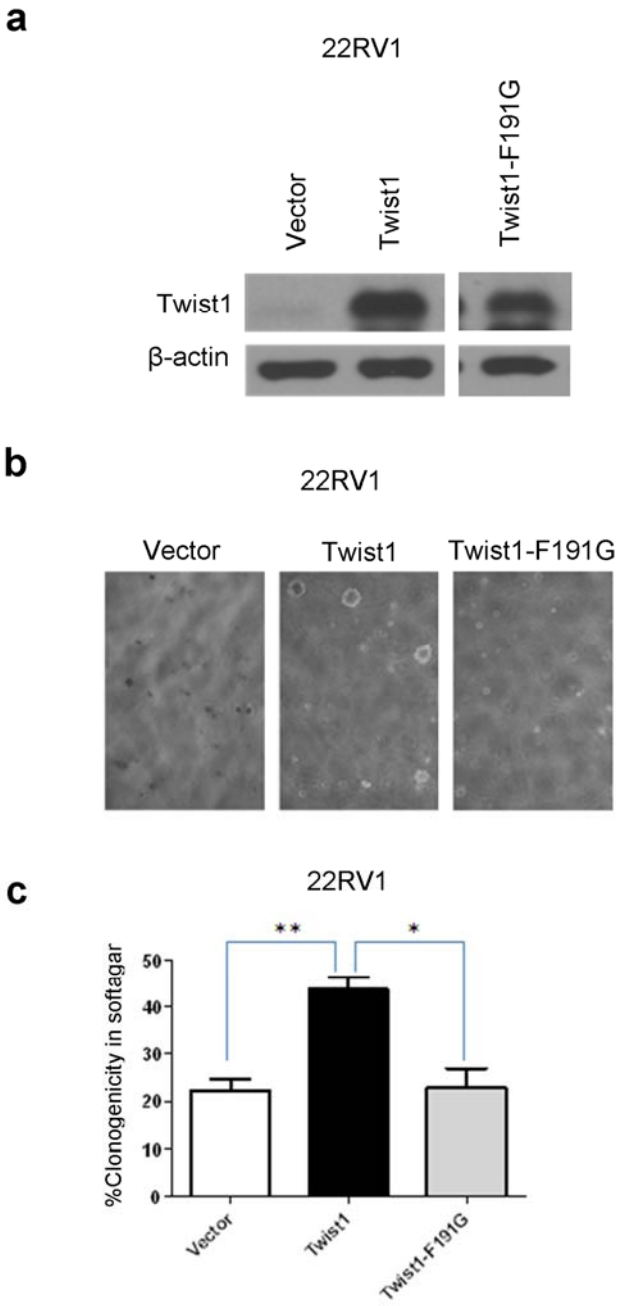
Supplementary Figure S6. The Twist box domain is required for full Twist1-induced cellular migration in PC3 cells. (**a)** Scratch wound healing assay was performed in PC3 isogenic cell lines and representative images shown at 0-hr and 24-hr. **(b)** Relative wound closure is calculated by the remaining wound area normalized to the initial wound area (n=3, 3 fields; * - p <0.05 and ** - p <0.01 by Mann-Whitney test) by ImageJ software (NIH) and showed that PC3 cells overexpressing Twist1-F191 cells were less migratory than wildtype Twist1 cells.



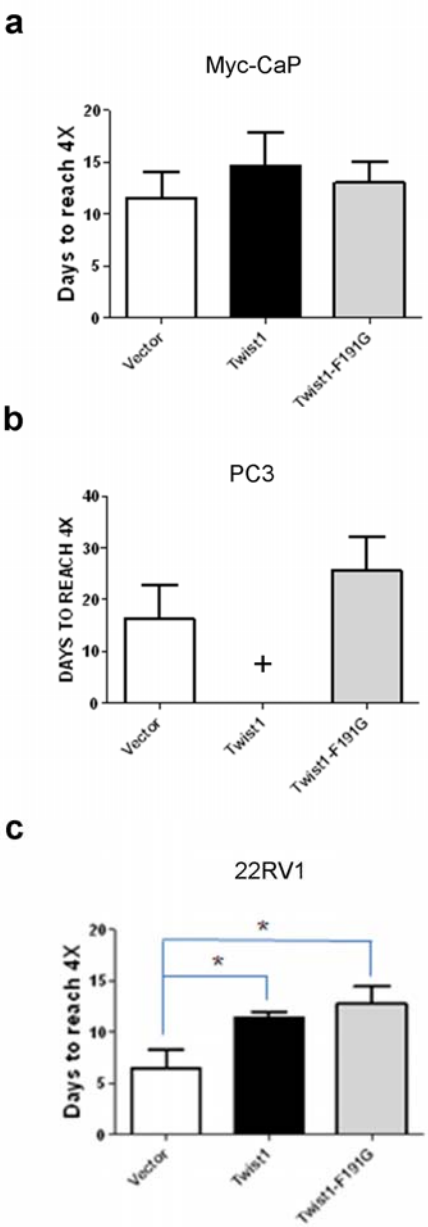
Supplementary Figure S7. Twist1 overexpression increases cell traction forces of individual androgen-independent PC3 prostate cancer cells. (**a**) The top panel shows representative phase contrast images of PC3 cells expressing Vector control and Twist1 (n=16 for each cell line). The bottom panel shows traction maps of the respective PC3 cells; the colors within the cells represent the absolute magnitude of tractions in Pascals, and the arrows represent the relative magnitude and directions. At the single cell level, Twist1 overexpressing PC3 cells exhibited a larger spreading area (**b**) and a higher cell traction force (**c**) than Vector control PC3 cells. Bars represent column mean; error bars are ± SEM. The values are significant by Mann Whitney test: ***, $p<0.001$.



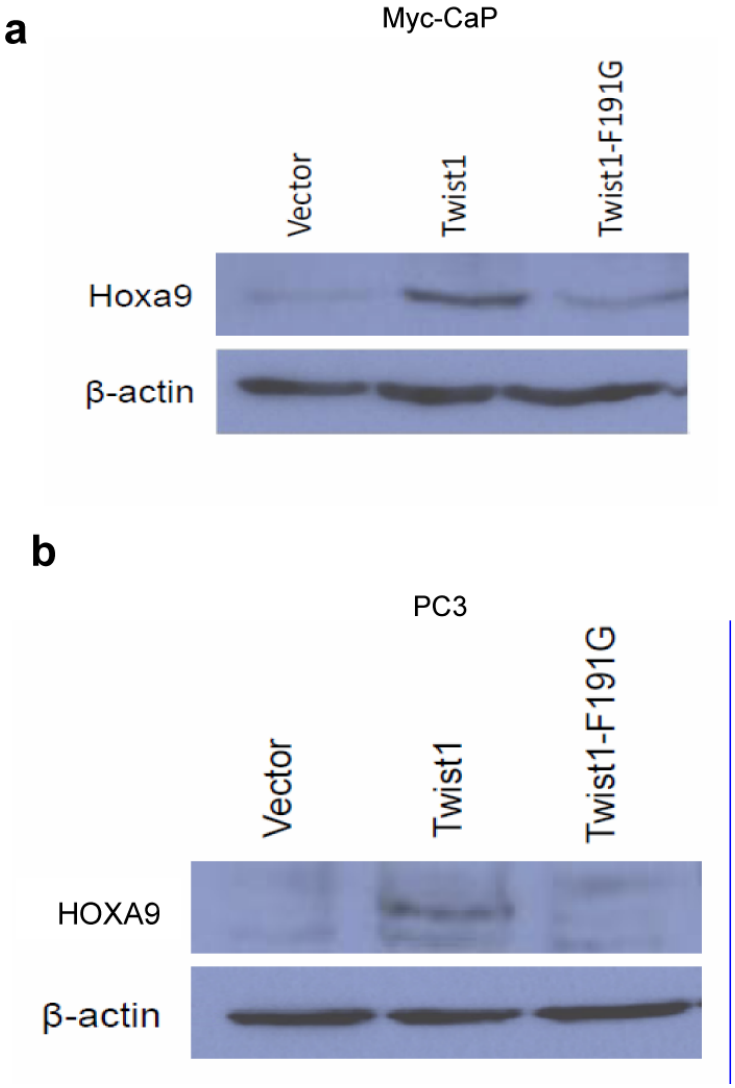
Supplementary Figure S8. Twist1 overexpression confers radioresistance to prostate cancer cells which is attenuated by mutation of the Twist box domain. Briefly, the clonogenic survival assay was performed (see Materials and Methods) by plating radiated single cells, 200-5000, in 100 mm dishes, stained 2-3 weeks later with crystal violet and distinct colonies (defined as ≥50 cells) scored. The survival fraction at 4 or 5 Gy is calculated by total number of colonies normalized to the plating efficiency. Survival fraction is plotted for (**a**) Myc-CaP (n=3, 5 replicates per experiment) and (**b**) PC3 isogenic cell lines (n=2, 5 replicates per experiment). Bars represent column mean; error bars ± SEM; Significance by Mann-Whitney test: *, $p < 0.05$; and **, $p < 0.01$.



Supplementary Figure S9. The Twist box domain is required for Twist1-induced soft agar anchorage-independent growth of 22Rv1 prostate cancer cells. (**a**) Western blot analysis of 22Rv1 cells stably overexpressing similar levels of Twist1 and Twist1-F191G. β-actin was used as internal control. (**b**) The representative phase contrast images of soft agar colonies from 22RV1 isogenic cells taken at 4X objective. (**c**) Colonies containing above 50 cells are scored in 5 random fields from each well (n=6) and percentage determined from the number of soft agar colonies normalized with the total number of cells. Bars represent column mean; error bars ± SEM; Significance is by Mann-Whitney test: *, $p < 0.05$; and **, $p < 0.01$.



Supplementary Figure S10. Twist1 overexpression does not confer prostate cancer cells increased primary tumorigenicity and slows primary tumor cell growth *in vivo*. Subcutaneous tumor xenografts were initiated by subcutaneously injecting isogenic cells into the flanks of mice. Tumors that reached 100 mm^3 were then measured three times a week and then scored for the time taken to reach 4X the tumor starting volume. Cohorts of 5 mice were used for each cell line. (**a**) 1 X 10^6 Myc-CaP isogenic cells were injected into FVB/N mice and no difference was seen between Vector, Twist1 or Twist1-F191G expressing cells for tumorigenicity or primary tumor growth *in vivo*. (**b**) 2 X 10^6 PC3 isogenic cells were injected into nude mice and stable Twist1 overexpression made PC3 cells less tumorigenic *in vivo*. + - indicates no subcutaneous tumors formed. PC3 cells stably overexpressing Twist1-F191G were able to form subcutaneous tumors suggesting the Twist box domain was required for Twist1-dependent inhibition of primary subcutaneous tumorigenicity *in vivo*. (**c**) 2 X 10^6 22RV1 isogenic cells were injected into nude mice and showed that Twist1 overexpression delayed primary tumor growth *in vivo* (*, $p < 0.05$ by Mann-Whitney test). Bars represent column mean; error bars ± SEM



Supplementary Figure S11. The Twist box domain is required for full Twist1-induced expression of Hoxa9/HOXA9. (**a)** Isogenic Myc-CaP cells show that Twist1 upregulates Hoxa9 by Western and requires the Twist box domain for this activity. (**b**) Similar results were seen for HOXA9 in isogenic PC3 cells by Western.