

SUPPLEMENTARY INFORMATION

Akt/PKB-mediated Phosphorylation of Twist1 Promotes Tumor Metastasis via Mediating Cross-talk Between PI3K/Akt and TGF β Signaling Axes

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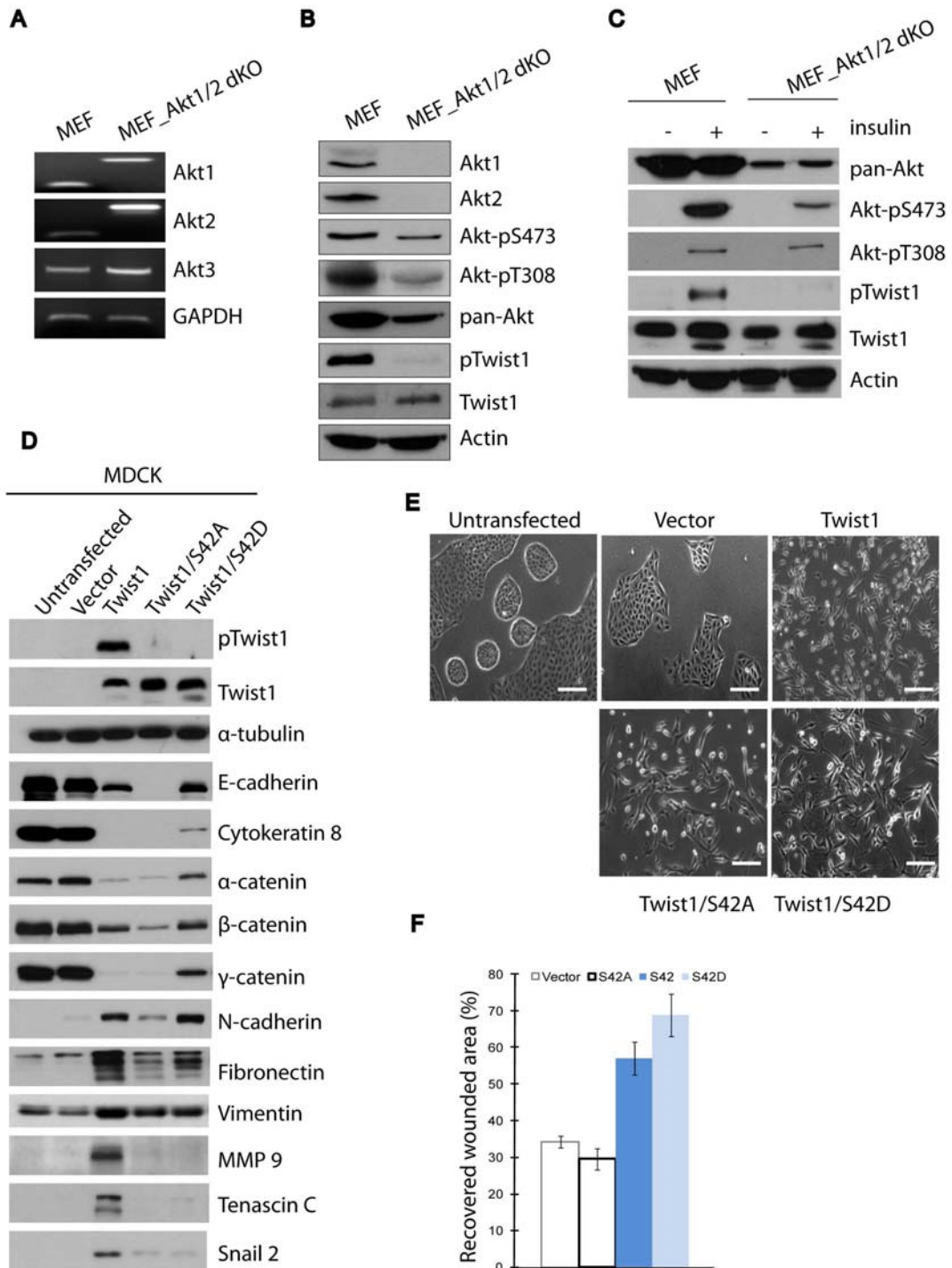


Figure S1. Twist1 phosphorylation is dependent on activated Akt1/Akt2 and promotes cell motility

A, The genetic background of Akt1/Akt2-dKO cells established and published by Morris J. Birnbaum's laboratory (University of Pennsylvania, the USA) was confirmed by genotyping (GT). The *GAPDH* gene was used as an internal control. **B**, Wild-type Twist1 was introduced into wild-type MEFs and Akt1/Akt2-dKO MEFs by retroviral infection. Individual Akt isoforms and Akt phosphorylated at both S473 and T308, and the expression and phosphorylation of Twist1 on S42 were investigated by immunoblotting. **C**, Twist1-expressing MEFs and Akt1/Akt2-dKO MEFs were cultured in serum-depleted medium for 16 h and subsequently stimulated by adding 10 μ M insulin for 30 min. Phosphorylation of Akt and Twist1 were analyzed by immunoblotting. Total cell lysates were prepared by adding the sampling buffer of SDS-PAGE. **D**, Twist1 and its variants were stably expressed in MDCK cells and the indicated epithelial and mesenchymal markers were analyzed by western blotting. **E**, Morphological changes of MDCK cells expressing Twist1 and its variants. **F**, Wound healing assay of MDCK cells expressing Twist1 and its variants.

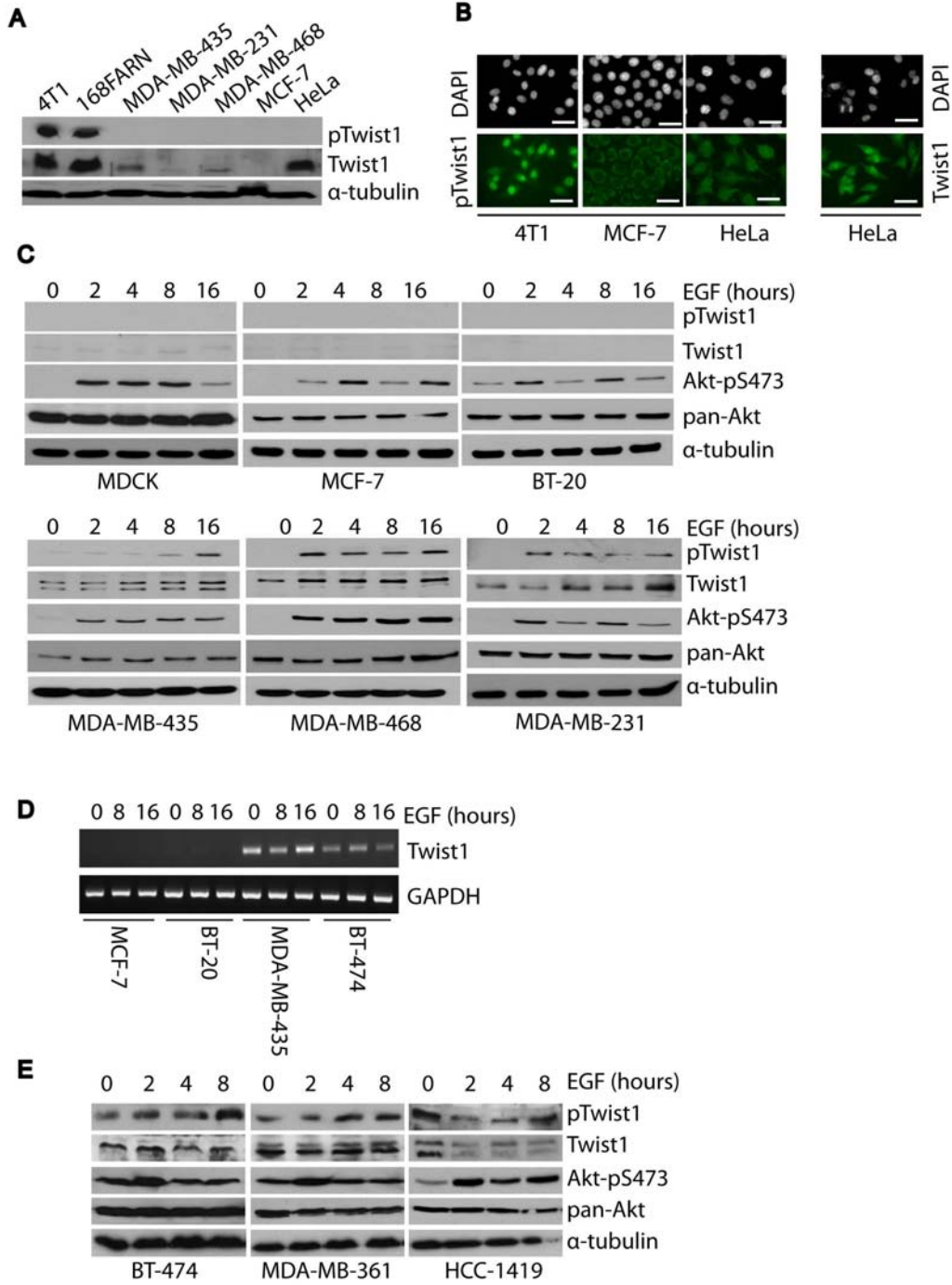


Figure S2. Twist1 is phosphorylated in invasive human breast cancer cell lines and tumors.

A-B, Two mouse metastatic breast cancer cell lines (4T1 and 168FARN) and several human breast cancer cell lines with different metastatic potential were used to investigate the status of Twist1 phosphorylation. Cells were cultured in standard DMEM containing 10% FBS, lysed in sample buffer and probed for pTwist1. Phosphorylated Twist1 was detected in invasive 4T1 cells by western blotting and immunofluorescence staining. Nuclear localization of pTwist1 and total Twist1 in 4T1 cells were detected by immunofluorescence staining. MCF-7 cells that do not express Twist1 were used as control for total Twist1. Although its expression has been frequently detected, endogenous Twist1 was not phosphorylated in HeLa cells. **C**, Twist1 phosphorylation in metastatic breast cancer cell lines MDA-MB-231, MDA-MB-468 and MDA-MB-435 could be stimulated by activation of EGFR pathway. Cells were serum-starved overnight and incubated with EGF (100 ng/ml) and lysed at different time points and crude lysate was subjected to immunoblotting analysis of Twist1 expression and phosphorylation. The untransformed cell line MDCK and non-metastatic breast cancer cell lines MCF-7 and BT-20 were used as controls. **D**, Analysis of Twist1 mRNA level after EGF stimulation in non-metastatic breast cancer cell lines MCF-7 and BT-20. Twist expressing metastatic cell lines MDA-MB-435 and BT-474 were used as controls. **E**, Western blotting analysis of Twist1 and Akt phosphorylation in HER2-high breast cancer cell lines treated with EGF at indicated time points.

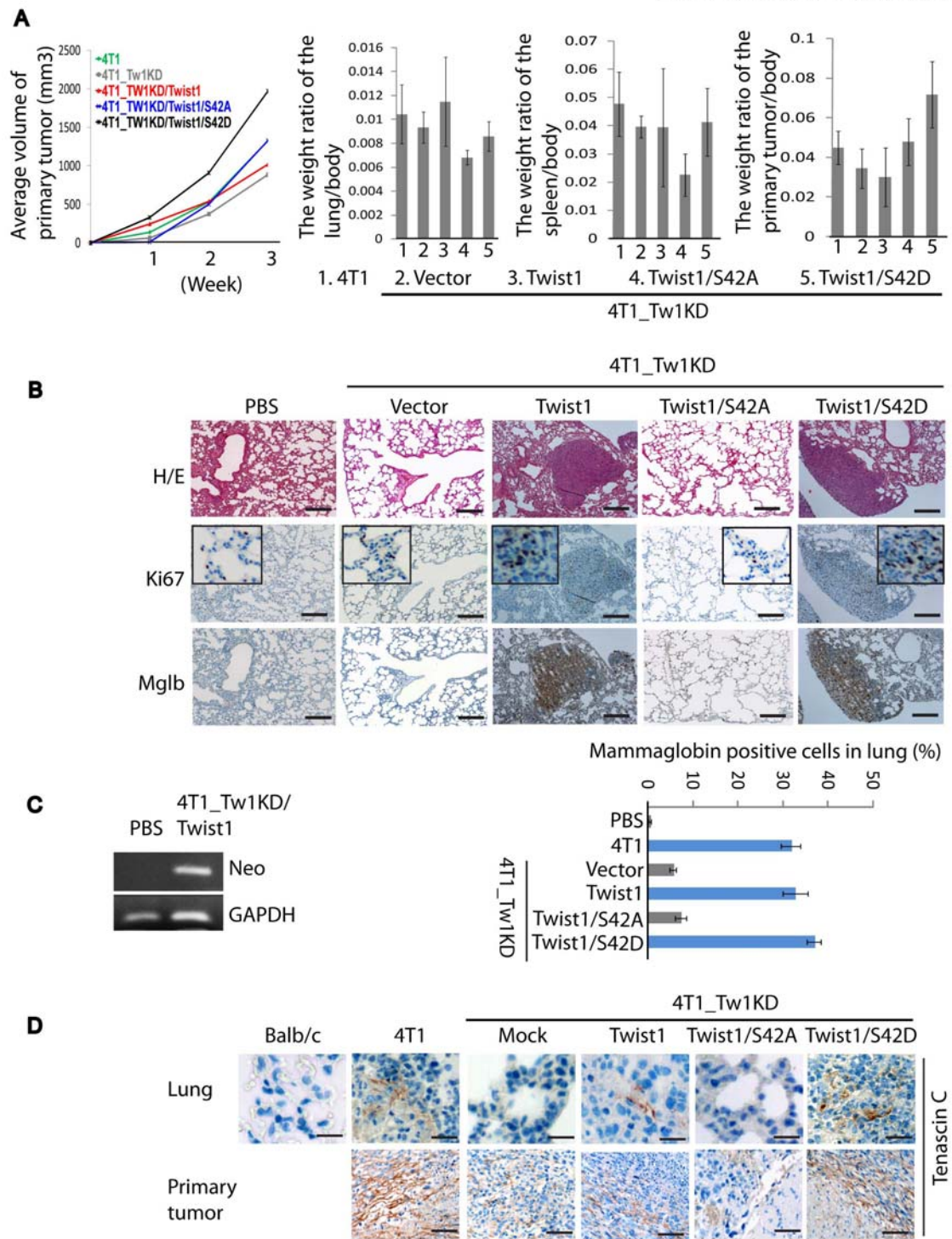
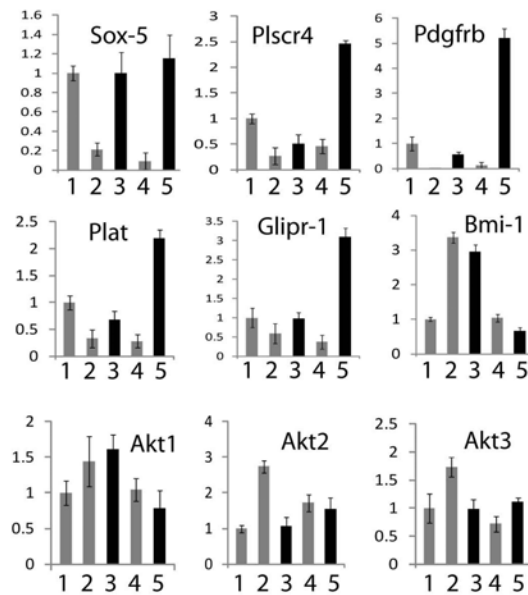
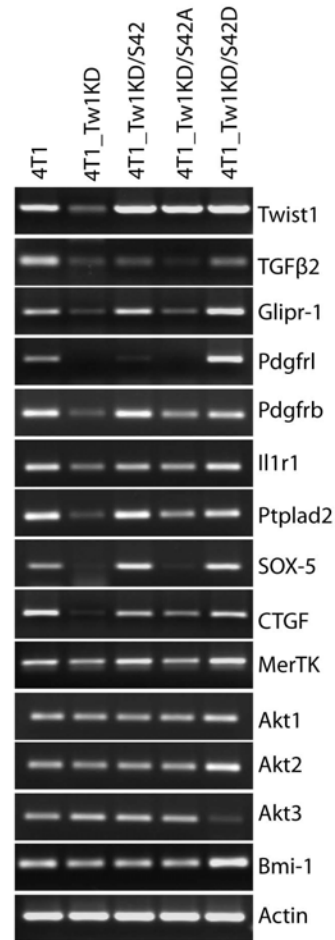


Figure S3. Phosphorylation of Twist1 promotes breast cancer metastasis *in vivo*.

A, The size of primary tumors, body weight and the weight of the lung and the kidney tissues were monitored. Tumors were measured weekly with calipers. Each group represents the average of 6 mice. **B**, Immunohistochemical Staining of Lung Metastases. Serial sections of lung tissue were stained for Ki67, mammaglobin and H&E. Statistic analysis of mammaglobin-positive cells in individual serial sections of lungs. Bar represent means \pm SEM of samples measured in triplicate. **C**, The gene encoding the Neomycin-cleaving enzyme in plasmid constructs was examined in lungs by PCR. The *GAPDH* gene was used as an internal control. **D**, Immunohistochemical staining of the invasive marker Tenascin C in primary tumors and lung tissues dissected from BALB/c mice injected with Twist1 and its variants.

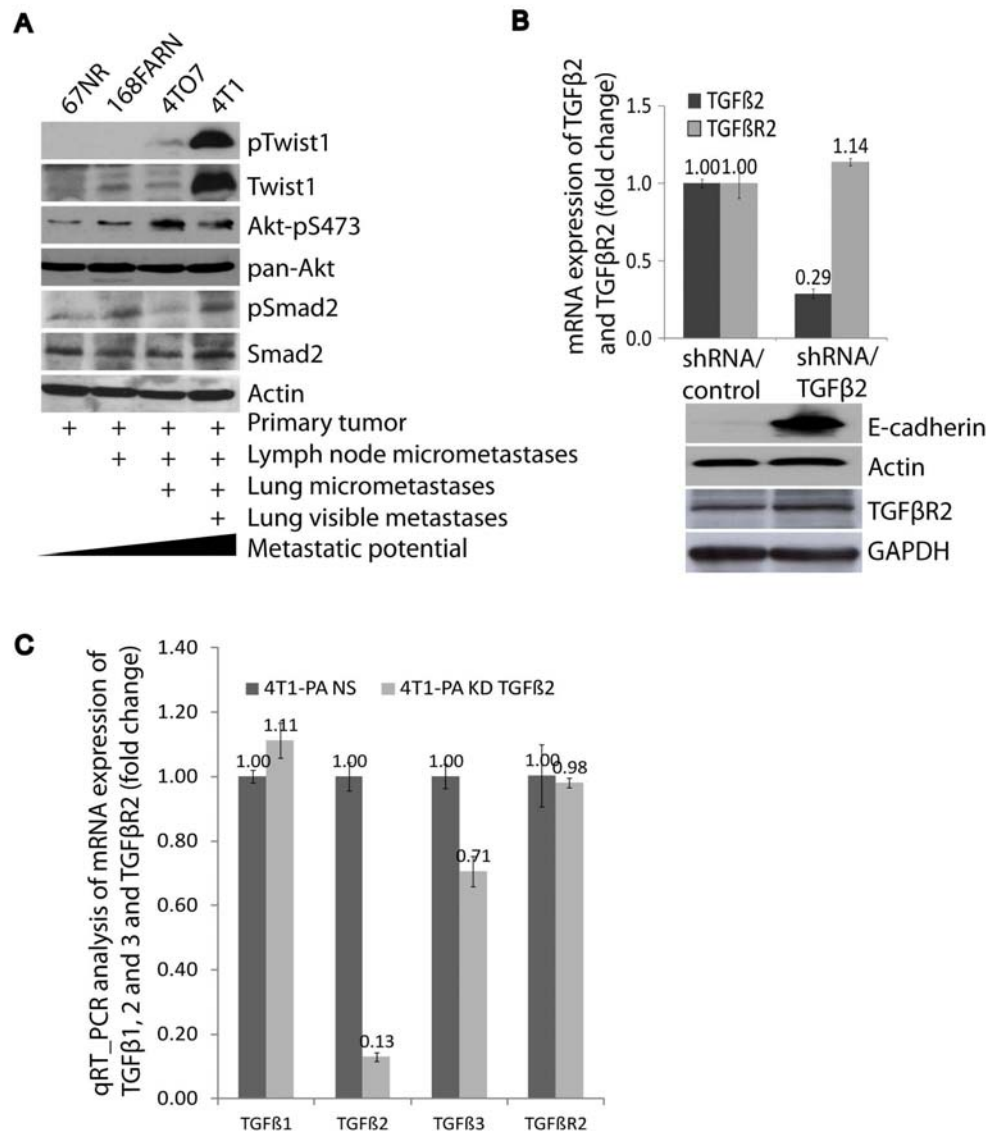
A**B****C**

qPCR primer sequences

Genes	Forward (5' - 3')	Reverse (5' - 3')
Twist1	GGACAAGCTGAGCAAGATTCA	CGGAGAAGGCGTAGCTGAG
Tgfb2	CTTCGACGTGACAGACGCT	GCAGGGGCAGTGTAACTTATT
Pdgfrb	TTCCAGGAGTGATACCAGCTT	AGGGGGCGTGATGACTAGG
Pdgfr1	ACTTCTGTTGCTACACGAAGC	CGGTTGAGTCAGTGGAGTCC
MerTK	ACCCAGTTGCTAGAGAGCTG	TGGTGAGTCTGTCTCCGGTAA
Glipr-1	CATCCTTGCTGTGATAGTCTGG	TTTGACCGAAGCTGGTTGTGA
Il1r1	GTGCTACTGGGGCTCATTGT	GGAGTAAGAGGACACTTGCGAAT
Ptplad2	AGCCCAGGTATAGGAAGAATGT	CCGCATAACTAATCCCAATAGCG
Sox-5	CCCGTGATCCAGAGCACTTAC	CCGCAATGTGGTTTTTCGCT
Plat	TTGTAGGGAAACGCTGTGACA	ACTGCTATTCCAGTTGATGCAC
Ctgf	CCCTAGCTGCCTACCGACT	CATTCCACAGGTCTTAGAACAGG
Bmi-1	ATCCCCACTTAATGTGTGCCT	CTTGCTGGTCTCCAAGTAACG
Akt1	ATGAACGACGTAGCCATTGTG	TTGTAGCCAATAAAGGTGCCAT
Akt2	ACGTGGTGAATACATCAAGACC	GCTACAGAGAAATTGTTCAAGGG
Akt3	TGGGTTTCAAGAGGGGAGAA	AGGGGATAAGGTAAGTCCACATC
Actin	GGCTGTATTCCCCTCCATCG	CCAGTTGGTAACAATGCCATGT

Figure S4. Validation of potential phospho-Twist1 targeting genes.

A, Quantitative PCR analysis of indicated genes that are potentially regulated by phosphorylated Twist1. The ratio of relative fold-change of mRNA (1: 4T1, 2: 4T1_Tw1KD/Mock, 3: 4T1_Tw1KD/Twist1, 4: 4T1_Tw1KD/Twist1/S42A, 5: 4T1_Tw1KD/Twist1/S42D) is shown after normalization to β -actin mRNA. Values and standard deviation for each group were calculated from triplicates samples. **B**, mRNA expression of the candidate genes. **C**, Primer sequences used for 16 tested genes by qRT-PCR.

**Figure S5.**

A, four mouse breast cancer cell lines isolated from the same BALB/c tumor with distinct metastatic potential were examined for expression and phosphorylation of Smad2, Twist1 and Akt. **B**, *tgfβ2* was knocked down by shRNA in 4T1 cells. mRNA expression of TGFβ2 and TGFβRII were validated by qPCR. Increased E-cadherin and unchanged TGFβRII expression was shown by western blot. **C**, qRT-PCR analysis of *tgfβ1* and *tgfβ3* in 4T1 cells with *tgfβ2* knockdown

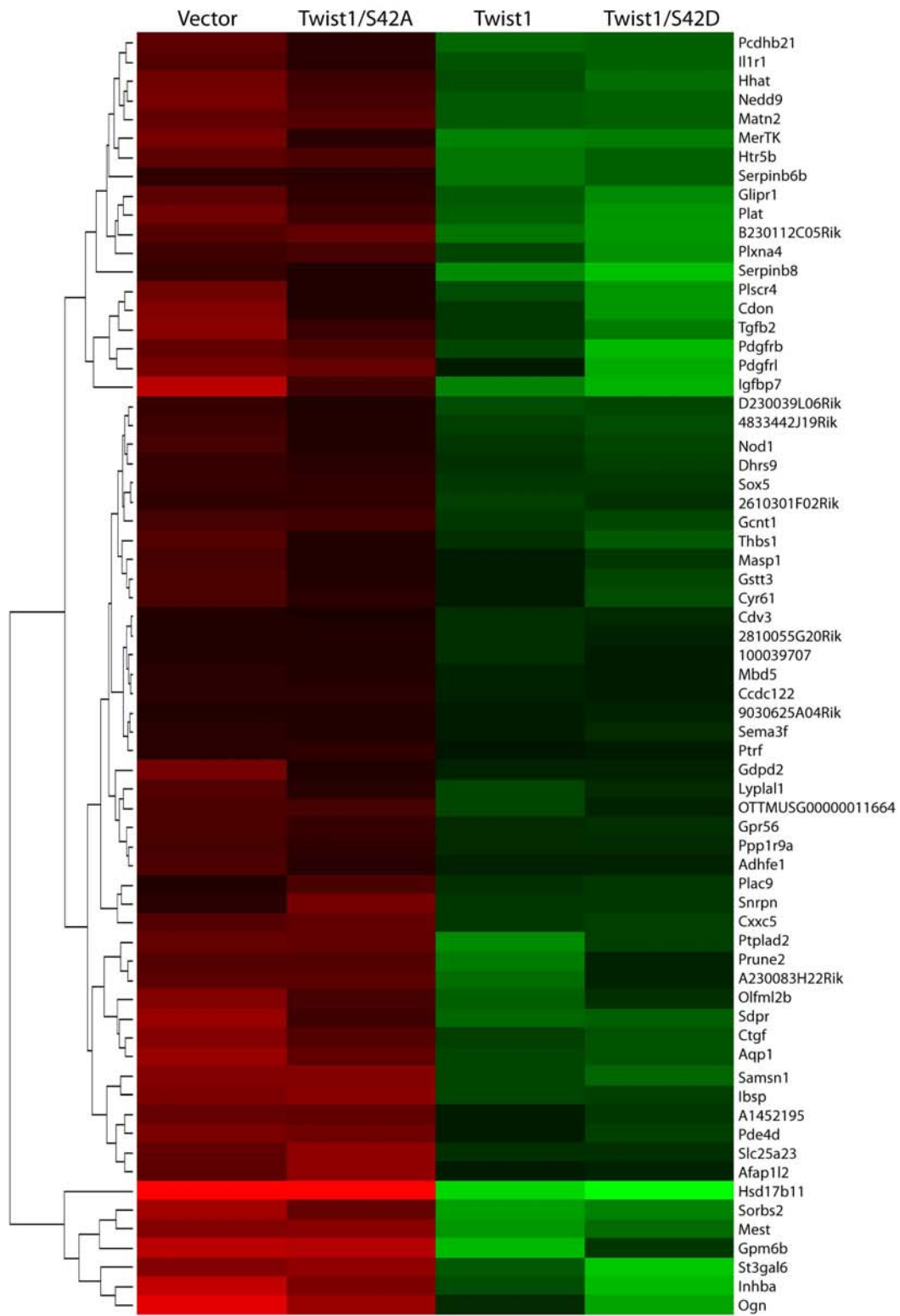
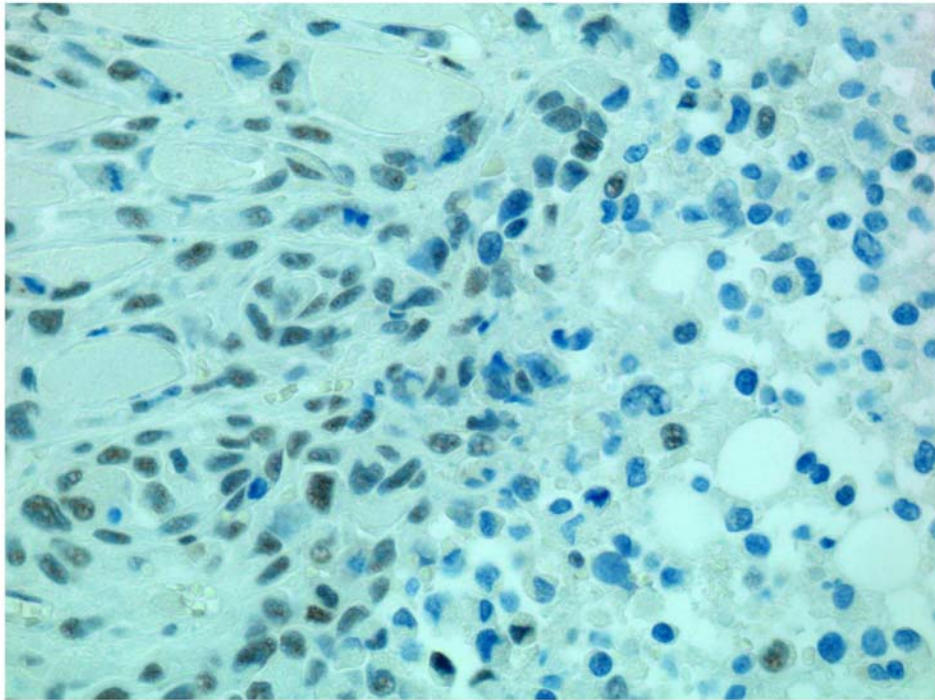


Figure S6. Heatmap of 68 genes that are potentially regulated by pTwist1.

MDA-MB-231 tumor



pTwist1

Figure S7. MDA-MB-231 xenograft tumor was stained for phospho-Twist1.
Figure S8. Staining of phospho-Twist1 and vimentin in the lung tissue of MDA-MB-231-injected mice (arrows indicate positive cells).

Sequences of primer pairs used in chromatin immunoprecipitation

Mouse TGFb2>mm9_dna range=chr1:188529771-188530871
Promoter sequence taken: -1000~+100.

	Forward	Reverse
(148bp):	ACAGGAGGGACTCAGGGAAG	CAGTAAGGGAGGGCGACTG
(139bp):	TCCACACCCCTCCAATGG	ACGATTCTGCCC GGAGCAG
(167bp):	CGTCAGGGATGGAGAGAAG	TCGCACCCCTCCCTAGCTTC
(120bp):	G TTCCTTTATTCAAAGC	ACAGGTTGTTTTTCATTAG
(150bp):	GTTATGATACTGTGTGAC	TGTTGCCTTCTTTATGTGAGC

Supplementary Table 1. Twist1 overexpression in human cancers (22 different human cancer types have been reported to overexpress Twist1 in pubmed)

Human cancer types	Twist1 Over-expression	Akt Amplification & mutation	References
Breast cancer (lobular carcinoma)	+	+	(Yang et al., 2004); (Watanabe et al., 2004)
Prostate cancer	+	+	(Kwok et al., 2005); (Li et al., 2008); (Wallerand et al., 2009)
Melanoma	+	+	(Hoek et al., 2004; Jinushi et al., 2008)
Pancreatic cancer	+	+	(Cates et al., 2009; Ohuchida et al., 2007; Satoh et al., 2008)
Primary Colorectal cancer	+	+	(Valdes-Mora et al., 2009)
Bladder cancer	+		(Wallerand et al., 2009; Zhang et al., 2007b)
Cervical cancer	+		(Shibata et al., 2008)
Choroid plexus papilloma	+		(Hasselblatt et al., 2009)
Rhabdomyosarcoma	+		(Maestro et al., 1999)
Neuroblastoma	+		(Valsesia-Wittmann et al., 2004)
Metastatic nasopharyngeal carcinoma	+		(Song et al., 2006; Zhang et al., 2007a)
Oesophageal squamous cell carcinoma	+		(Yuen et al., 2007)
Esophageal squamous cell carcinoma	+		(Xie et al., 2009)
Epithelial ovarian carcinoma	+	+	(Hosono et al., 2007; Kajiyama et al., 2006; Terauchi et al., 2007)
Infiltrative endometrial cancer	+		(Kyo et al., 2006; Tsukamoto et al., 2007)
Hepatocellular carcinoma	+		(Lee et al., 2006; Matsuo et al., 2009; Niu et al., 2007; Yang et al., 2009)
Gastric cancer (diffuse-type)	+	+	(Rosivatz et al., 2002; Yan et al., 2007)
Glioma	+	+	(Elias et al., 2005)
Pheochromocytoma	+		(Waldmann et al., 2009)
Parathyroid carcinoma	+		(Fendrich et al., 2009)
Spindle cell carcinoma of head and neck	+	+	(Kojc et al., 2009; Ou et al., 2008)
Metastatic pediatric osteosarcoma	+		(Entz-Werle et al., 2007)

Supplementary Table 2. 68 genes are potential targets of phospho-Twist1

Name	Accession	Gene_symbol	Description
10344725	NM_175236	Adhfe1	alcohol dehydrogenase, iron containing, 1
10345762	NM_008362	Il1r1	interleukin 1 receptor, type I
10346164	NM_138741	Sdpr	serum deprivation response
10349174	NM_011459	Serpinb8	serine (or cysteine) peptidase inhibitor, clade B, member 8
10351491	NM_177068	Olfml2b	olfactomedin-like 2B
10352143	BC120791	D230039L06Rik	RIKEN cDNA D230039L06 gene
10357288	NM_010483	Htr5b	5-hydroxytryptamine (serotonin) receptor 5B
10360914	NM_146106	Lyplal1	lysophospholipase-like 1
10360920	NM_009367	Tgfb2	transforming growth factor, beta 2
10361169	NM_144881	Hhat	hedgehog acyltransferase
10362201	NM_010217	Ctgf	connective tissue growth factor
10369993	NM_133994	Gstt3	glutathione S-transferase, theta 3
10372410	NM_028608	Glipr1	GLI pathogenesis-related 1 (glioma)
10391332	NM_008986	Ptrf	polymerase I and transcript release factor
10403743	NM_008380	Inhba	inhibin beta-A
10404422	NM_011454	Serpinb6b	serine (or cysteine) peptidase inhibitor, clade B, member 6b
10405063	NM_008760	Ogn	osteoglycin
10406782	ENSMUST00000042517	B230112C05Rik	RIKEN cDNA B230112C05 gene
10407097	NM_011056	Pde4d	phosphodiesterase 4D, cAMP specific
10407124	ENSMUST00000099179	AI452195	expressed sequence AI452195
10408850	NM_001111324	Nedd9	neural precursor cell expressed, developmentally down-regulated gene 9
10416533	NM_175369	Ccdc122	coiled-coil domain containing 122
10418205	NM_207229	Plac9	placenta specific 9
10421697	BC116748	9030625A04Rik	RIKEN cDNA 9030625A04 gene
10423599	NM_016762	Matn2	matrilin 2
10436596	ENSMUST00000068704	2810055G20Rik	RIKEN cDNA 2810055G20 gene
10438708	NM_008555	Masp1	mannan-binding lectin serine peptidase 1
10440099	NM_018784	St3gal6	ST3 beta-galactoside alpha-2,3-sialyltransferase 6
10440393	NM_023380	Samsn1	SAM domain, SH3 domain and nuclear localization signals, 1
10452257	NM_025877	Slc25a23	solute carrier family 25 (mitochondrial carrier; phosphate carrier), member 23
10454851	NM_133687	Cxxc5	CXXC finger 5
10455135	NM_053146	Pcdhb21	protocadherin beta 21
10456046	NM_008809	Pdgfrb	platelet derived growth factor receptor, beta polypeptide
10461869	ENSMUST00000067439	Prune2	prune homolog 2 (Drosophila)

10461878	BC094224	A230083H22Rik	RIKEN cDNA A230083H22 gene
10466521	NM_173442	Gcnt1	glucosaminyl (N-acetyl) transferase 1, core 2
10468668	NM_146102	Afap12	actin filament associated protein 1-like 2
10469609	ENSMUST000000100373	OTTMUSG00000011664	predicted gene, OTTMUSG00000011664
10471967	NM_029924	Mbd5	methyl-CpG binding domain protein 5
10472538	NM_175512	Dhrs9	dehydrogenase
10474700	NM_011580	Thbs1	thrombospondin 1
10475890	NM_008587	Mertk	c-mer proto-oncogene tyrosine kinase
10484197	ENSMUST000000049544	2610301F02Rik	RIKEN cDNA 2610301F02 gene
10484201	ENSMUST000000049544	2610301F02Rik	RIKEN cDNA 2610301F02 gene
10502655	NM_010516	Cyr61	cysteine rich protein 61
10510167	AF320340	Cdv3	carnitine deficiency-associated gene expressed in ventricle 3
10514275	NM_025760	Ptplad2	protein tyrosine phosphatase-like A domain containing 2
10523701	NM_008318	Ibsp	integrin binding sialoprotein
10530841	NM_008048	Igfbp7	insulin-like growth factor binding protein 7
10531919	NM_053262	Hsd17b11	hydroxysteroid (17-beta) dehydrogenase 11
10536297	NM_181595	Ppp1r9a	protein phosphatase 1, regulatory (inhibitor) subunit 9A
10537062	NM_008590	Mest	mesoderm specific transcript
10538459	NM_007472	Aqp1	aquaporin 1
10542880	BC048711	4833442J19Rik	RIKEN cDNA 4833442J19 gene
10543802	NM_175750	Plxna4	plexin A4
10544891	NM_172729	Nod1	nucleotide-binding oligomerization domain containing 1
10549200	NM_011444	Sox5	SRY-box containing gene 5
10564211	NM_013670	Snrpn	small nuclear ribonucleoprotein N
10570855	NM_008872	Plat	plasminogen activator, tissue
10571467	NM_026840	Pdgfrl	platelet-derived growth factor receptor-like
10571567	NM_172752	Sorbs2	sorbin and SH3 domain containing 2
10574259	NM_018882	Gpr56	G protein-coupled receptor 56
10584208	NM_021339	Cdon	cell adhesion molecule-related
10587818	NM_178711	Plscr4	phospholipid scramblase 4
10595630	NM_001128601	100039707	predicted gene, 100039707
10596747	NM_011349	Sema3f	sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3F
10601044	NM_023608	Gdpd2	glycerophosphodiester phosphodiesterase domain containing 2
10603151	NM_023122	Gpm6b	glycoprotein m6b