**SUPPLEMENTARY MATERIALS**

# Phosphorylation of Serine 68 of Twist1 by MAPKs Stabilizes Twist1 Protein and Promotes Breast Cancer Cell Invasiveness

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## Supplementary Methods:

***Antibodies and Western blotting.*** Antibodies against p38, p-p38, JNK, p-JNK, p44/42 ERKs, p-p44/42 ERKs and HA tag were purchased from Cell Signaling Technology. Antibodies against E-cadherin, N-cadherin, vimentin and β/γ-catenins were purchased from BD Transduction Laboratories. Antibodies against Flag tag, α-tubulin, and β-actin were purchased from Sigma. Antibodies against phosphorylated serine (p-Ser) and tyrosine (p-Tyr) were purchased from Abcam. The H-Ras antibody was from Santa Cruz. The antibody specific to the phosphorylated Serine 68 in Twist1 (pS68-Twist1) and the phosphorylation-insensitive Twist1 antibody were custom made by the Proteintech Group, Inc. Western blot was performed with the above antibodies by following standard protocols. The band intensities on X-ray films were measured by densitometry when semi-quantitative analysis was desirable.

***In vitro de-phosphorylation and phosphorylation assays.*** For de-phosphorylation assays, F-Twist1 immunoprecipitated from HEK293 cells in the presence of $NaVO_3$ was incubated with 0.2 U/ml of active λ protein phosphatase (λ-PPase) (New England BioLabs) or heat-inactivated λ-PPase in a phosphotase buffer for 30 minutes at 30$^{o}$C. Reactions were terminated by heating in the Laemmli sample buffer, and the Twist1 protein was analyzed by Western blotting. For phosphorylation assays, glutathione S transferase (GST) and GST-Twist1-N fusion proteins were expressed in BL21 *E. coli*, solubilized in a sarkosyl solusion as described (1) and purified using the glutathione-sepharose 4B breads (Amersham Pharmacia Biotech). The purified proteins were separately incubated with p38, ERK1, ERK2 and JNK kinases (Upstate) in a phosphorylation buffer containing ATP, and the reactions were carried out as described (2). The phosphorylation of Ser 68 in Twist1 was assayed by Western blotting with S68-Twist1-specific antibody.

***Cycloheximide (CHX)-based protein stability assay.*** The human Twist1 and S68A-Twist1 cDNAs were cloned into the pAB-HA vector between the Bam H1 and Hind III digestion sites. HEK293T cells were transfected with pAB-HA-Twist1 and pAB-HA-S68A-Twist1 vectors using Lipofectamin 2000. After 24 hours, cells were treated with 0.1 mg/ml of CHX and harvested at different time points. For inducible HEK293 cell lines, Twist1 and S68A-Twist1 proteins were induced by DOX treatment for 6 hours before CHX was added. To study the effect of H-RasV12 on Twist1, HEK293T cells were co-transfected with pAB-HA-Twist1 or pAB-HA-S68A-Twist1 plasmids and H-RasV12 expression plasmid or mock vector. Cells were harvested and analyzed by Western blotting. Band intensities were semi-quantitatively analyzed by densitometry. The degradation curve was plotted using the time period of CHX treatment for the x-axis and protein band intensities in logarithm for the y-axis. The linear regression was calculated using Microsoft Exel software as described (3). The protein half-life was determined as previously described (4).

***Ubiquitination Assay.*** The inducible F-Twist1 and F-S68A-Twist1 HEK293 cell lines were transfected with HA-tagged ubiquitin (HA-Ub) expression or mock plasmids using Lipofectamin 2000. After 24 hours, cells were treated with DOX for 6 hours to induce Twist1 and S68-Twist1 expression. Then, cells were treated with or without MG132 for another 6 hours. Clear cell lysates were prepared and immunoprecipitation was carried out using the anti-Flag M2 ararose beads at 4°C. After washing three times with lysis buffer, bound proteins were eluted and assayed by Western blotting using anti-HA and anti-Flag monoclonal antibodies.

***Semi-quantitative and quantitative RT-PCR.*** Total RNA was isolated from cells using TRIzol reagents (Invitrogen). Reverse transcription was performed with 1 μg RNA with a reverse transcriptase core kit

(Eurogentec). For semi-quantitative RT-PCR, the primer pairs of 5'-ctcggtctggaggatggag and 5'-cacgccctgtttctttgaat were used to detect Twist1 cDNA, while the primer pairs of 5'-gcaccggtggagcgatgacggaatat and 5'- agtgaattc gtcaggagagcacacac were used to detect H-Ras cDNA. For real-time PCR, the concentrations of human Twist1 cDNA were measured using specific TaqMan probes and gene-specific primers as described (5). The measurement of β-actin cDNA concentration served as an endogenous control for normalizing relative levels of Twist1 cDNA.

***Generation of MCF-10A cell lines.*** The H-RasV12 cDNA was cloned into the PQCXIN retroviral plasmid between the Age I and Eco R1 sites. The F-Twist1, F-S68A-Twist1 and F-S68E-Twist1 cDNAs were cloned into the PQCXIH retroviral plasmid between the Not I and Pac I sites. The 293T cells were co-transfected with the retroviral packaging plasmid, pCL-Ampho and each type of retroviral PQCX-based plasmid using Lipofectamine 2000. After 6 hours, the medium was replaced with fresh DMEM and cells were grown for an additional 24 hours. The medium containing retroviruses was collected and filtered through a 45-μm pore-size filter. MCF-10A cells were incubated overnight with each type of media containing PQCXIH-F-Twist1, -F-S68A-Twist1, -F-S68E-Twist1 or -mock retroviruses. The media was supplemented with 10 μg/ml of polybrene. Forty-eight hours later, medium was replaced with selection medium containing 400 μg/ml of Hygromycin. The cultures were maintained in the selection medium for 10-14 days to establish stable cell pools. The cell pools were further infected using the PQCXIN-H-RasV12 or mock retroviruses to generate stable cell pools expressing either Twist1 or H-RasV12, or both.

***Real-Time Assays of Cell Invasion.*** Real-time cell invasion assay was carried out using an RT-CES system (ACEA Biosciences) as described (5). In this system, the cells in the upper chamber penetrate a Matrigel layer into the lower chamber. The invaded cell number/density in the lower chamber was reflected by the increase in current resistance and the data were presented as cell number/density index. For TGF-β promoted invasion assay, MCF-10A cells were pre-treated and treated with or without TGF-β1 (10 ng/ml) before and after they were seeded onto the Matrigel-coated top chambers.

***Analysis of breast tumor specimens.*** A total of 24 human breast tumor specimens were collected from surgically removed tumor tissues from breast cancer patients at Luzhou Medical College Affiliated Hospital in the year of 2009. All 24 patients were Asian women and aged 33-65 years old. No patient survival data were available at this stage. A portion of the specimen was used for clinical diagnosis of tumor pathology and immunohistochemistry for ERα, PR, HER2, and Ki67. The remaining tumor tissues were immediately frozen in liquid nitrogen and stored at -80$^{o}$C. Tumor tissue lysates were prepared after homogenizing the tissues in a lysis buffer containing the protease inhibitor cocktail and the $NaVO_3$ phosphotase inhibitor. The tissue lysates were used in Western blotting analysis of pS68-Twist1, Twist1 and p-JNK, p-p44/42 ERKs and p-p38 MAPKs. Band intensities were determined by densitometry and normalized to β-actin band intensity. Correlation degrees between two protein levels were determined by Spearman correlation analysis.

**References:**


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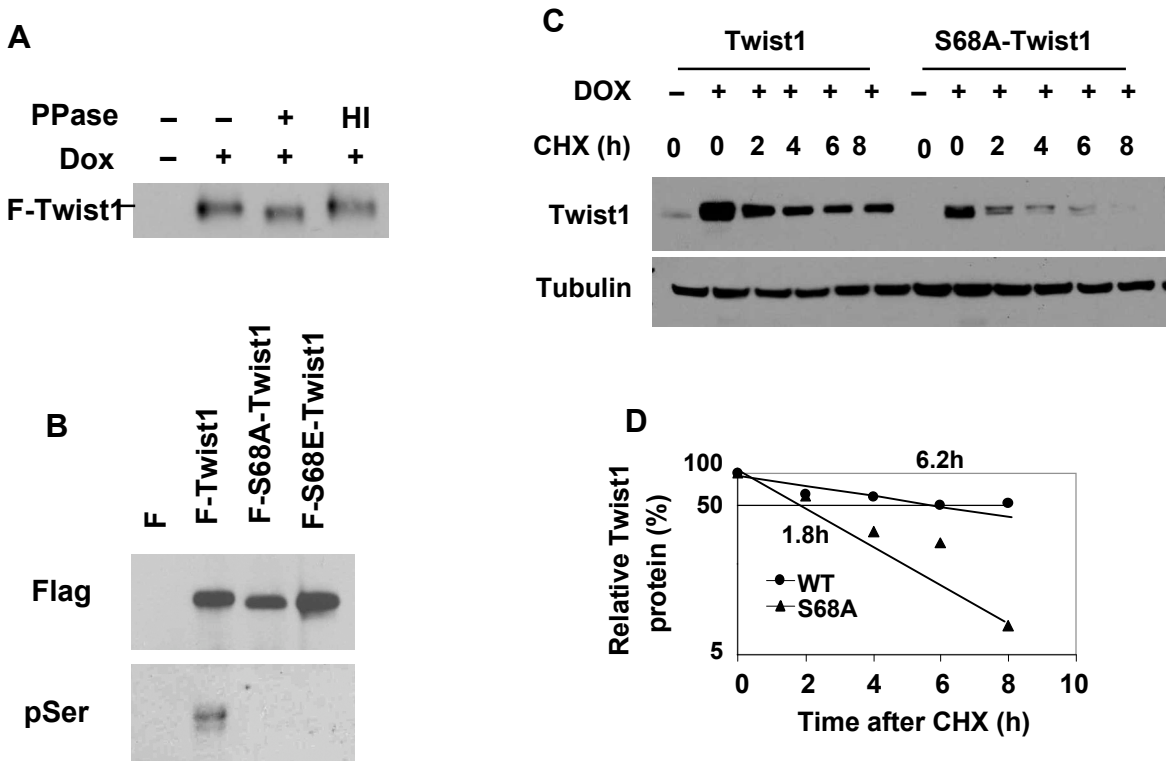
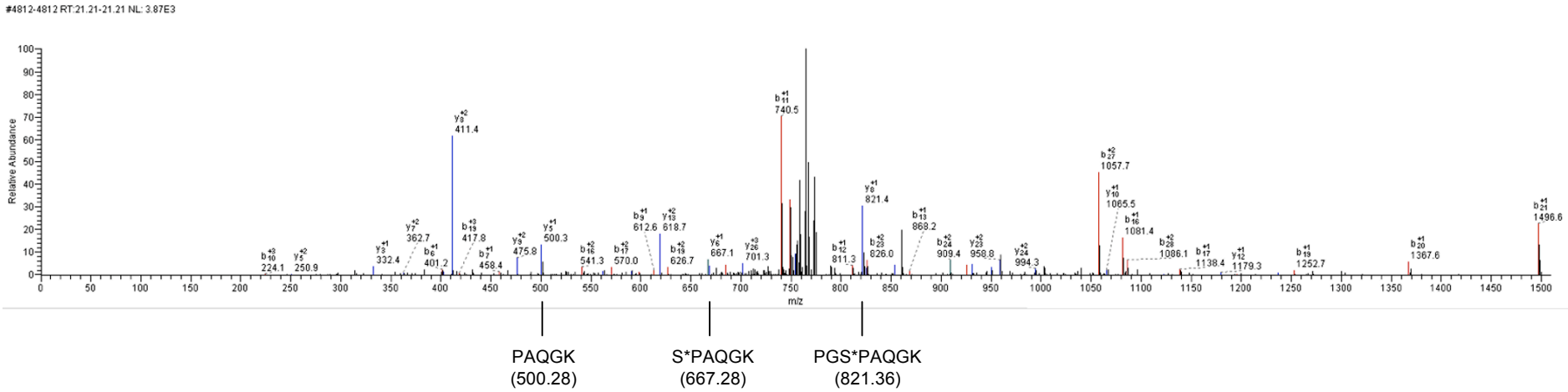
4. Belle, A., Tanay, A., Bitincka, L., Shamir, R., and O'Shea, E. K. Quantification of protein half-lives in the budding yeast proteome. Proc Natl Acad Sci U S A, *103:* 13004-13009, 2006.
5. Qin, L., Liu, Z., Chen, H., and Xu, J. The steroid receptor coactivator-1 regulates twist expression and promotes breast cancer metastasis. Cancer Res, *69:* 3819-3827, 2009.



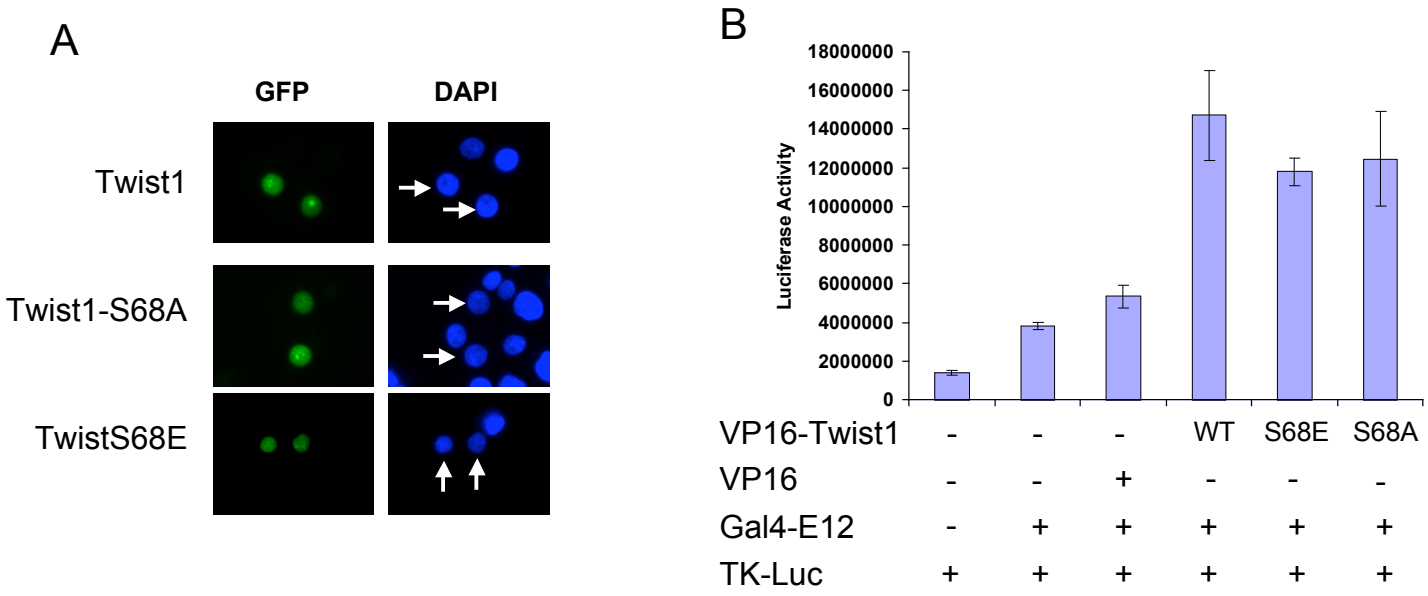

**Supplementary Fig. S1. Twist1 is a phosphoprotein and mutation of Serine 68 into alanine decreases Twist1 stability.** ***A.*** The inducible HEK293 cells for F-Twist1 expression were treated with vehicle (-) or Dox. F-Twist1 was immunoprecipitated and subjected to dephosphorylation assays. Immunoblotting analysis was performed with Flag antibody. HI, heat inactivated PPase. ***B.*** The indicated proteins were immunoprecipitated from inducible HEK293 cells expressing F, F-Twist1, F-S68A-Twist1 and F-S68E-Twist1 and analyzed by immunoblotting with Flag and anti-pSer antibodies. ***C.*** Twist1 and S68A-Twist1 inducible HEK293 cells were treated with DOX for 6 hours, and then with cycloheximide for the time period indicated. Immunoblotting was performed with antibodies against Flag and tubulin. ***D.*** The intensities of F-Twist1 and F-S68A-Twist1 bands detected by immunoblotting were determined by densitometry, and the data in natural logarithm are plotted against cycloheximide treatment time. The half lives of Twist1 and S68A-Twist1 determined by the degradation curves were 1.8 and 6.2 hours, respectively.



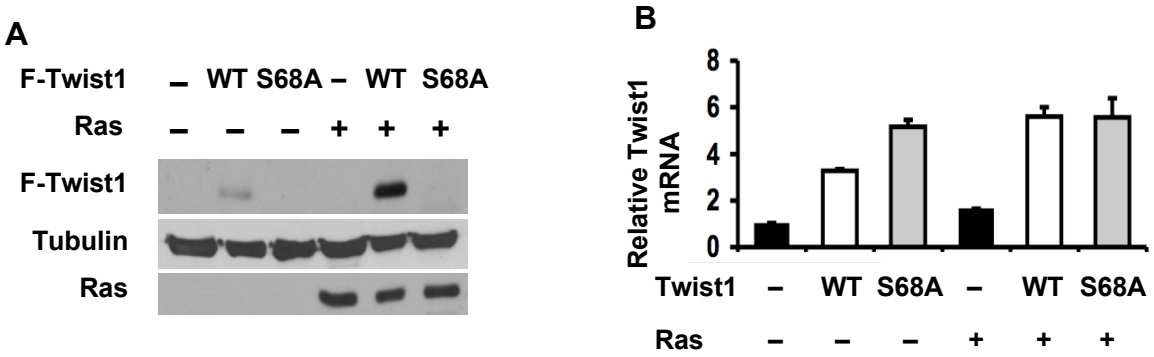

| # | AA | B (Mass) | Y (Mass) | # | |
|---|---|---|---|---|---|
| 1 | S | 88.04 | - | 29 | |
| 2 | A | 159.08 | 2229.95 | 28 | |
| 3 | G | 216.10 | 2158.91 | 27 | |
| 4 | G | 273.12 | 2101.89 | 26 | |
| 5 | G | 330.14 | 2044.87 | 25 | |
| 6 | A | 401.18 | 1987.85 | 24 | |
| 7 | G | 458.20 | 1916.81 | 23 | |
| 8 | P | 555.25 | 1859.79 | 22 | |
| 9 | G | 612.27 | 1762.74 | 21 | |
| 10 | G | 669.30 | 1705.72 | 20 | |
| 11 | A | 740.33 | 1648.70 | 19 | |
| 12 | A | 811.37 | 1577.66 | 18 | |
| 13 | G | 868.39 | 1506.62 | 17 | |
| 14 | G | 925.41 | 1449.60 | 16 | |
| 15 | G | 982.43 | 1392.58 | 15 | |
| 16 | V | 1081.50 | 1335.56 | 14 | |
| 17 | G | 1138.52 | 1236.49 | 13 | |
| 18 | G | 1195.55 | 1179.47 | 12 | |
| 19 | G | 1252.57 | 1122.45 | 11 | |
| 20 | D | 1367.59 | 1065.42 | 10 | |
| 21 | E | 1496.64 | 950.40 | 9 | |
| 22 | P | 1593.69 | 821.36 | 8 | |
| 23 | G | 1650.71 | 724.30 | 7 | |
| 24 | S* | 1817.71 | 667.28 | 6 | ← Phosphorylated |
| 25 | P | 1914.76 | 500.28 | 5 | |
| 26 | A | 1985.80 | 403.23 | 4 | |
| 27 | Q | 2113.86 | 332.19 | 3 | |
| 28 | G | 2170.88 | 204.13 | 2 | |
| 29 | K | - | 147.11 | 1 | |

**Supplementary Fig. S2. A collision induced fragmentation spectrum that identifies S68 in Twist1 as the phosphorylation site**. The observation of the y5 (m/z 550.3) and y6 ions (m/z 667.1) unambiguously identified S68 as the phosphorylated serine residue. The assignments of other observed ions to the calculated values further confirm this result.

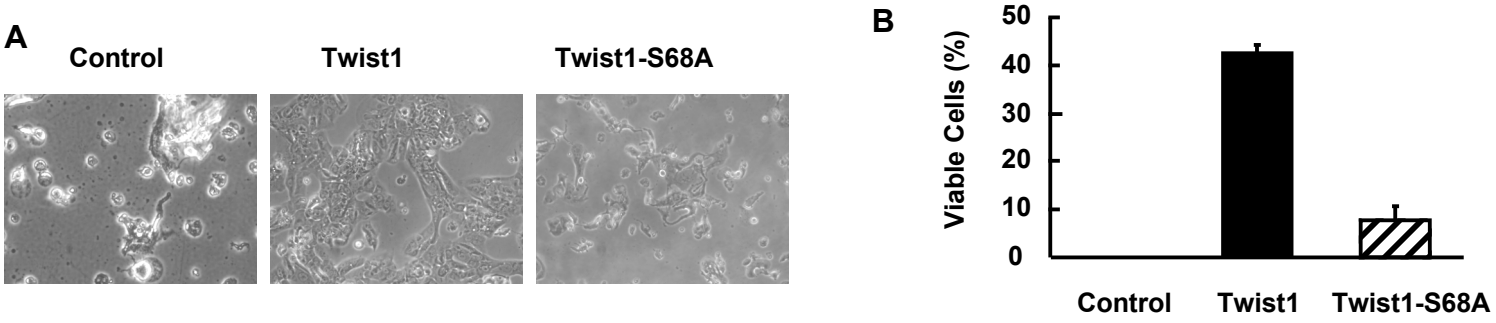
Assay conditions:
DTA for scan: 4812
Precursor ion: 773.72
Charge: 3
Activation type: CID
Mass type: mono
Mod's: (STY*+79.96633)(K#+42.01057)
(M@+15.99492)(C^+7100000)




**Supplemental Fig. S3. Serine 68 mutation does not affect Twist1 subcellular localization and dimerization with E12.** ***A.*** Twist1 and Twist1-S68A GFP fusion proteins were expressed in transfected Hela cells, and the cell nuclei were stained with DAPI. Stained cells were imaged under a fluorescence microscope. ***B***. Mammalian two-hybrid system assay for heterodimerization between E12 and Twist1 or Twist1 mutants. The 293 cells were transfected with the pGL3-UAS-tk-luciferase Gal 4 reporter and Gal-DBD-E12 expression plasmids in combination with VP16-Twist1, VP16-Twist1-S68A, or VP16-Twist1-S68E expression plasmid. RSV-β-Galactosidase plasmid was co-transfected as a normalizer of tranfection efficiency. Luciferase activity was assayed 40 hours after transfection and normalized to the β-galactosidase activity.




**Supplementary Fig. S4. H-RasV12 expression increases the stability of Twist1 protein** ***A.*** Stable MCF10A cell lines expressing F-Twist1, F-S68A-Twist1 and/or H-RasV12 or with their mock vectors as controls as indicated were generated. Lysate of each cell line was assayed by immunoblotting with antibodies against Flag, α-tubulin and H-Ras. ***B.*** Total RNA of each cell line was isolated. The Twist1 and S68A-Twist1 mRNA levels were assayed by real time RT-PCR and the relative expression levels were normalized to 18 S RNA.




**Supplementary Fig. S5. The S68 phosphorylation site of Twist1 is required for taxol resistance.** Stable MCF-10A cell lines with mock control, Twist1 expression or Twist1-S68A expression vectors were treated with 4 ug/ml of taxol for 6 days. Images were taken by phase contrast microscopy (Panel A). Number of viable cells were counted and normalized to the number of plated cells (Panel B).