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

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Abstract

Twist1, a basic helix–loop–helix transcription factor, promotes breast tumor cell epithelial–mesenchymal transition (EMT), invasiveness, and metastasis. However, the mechanisms responsible for regulating Twist1 stability are unknown in these cells. We identified the serine 68 (Ser 68) as a major phosphorylation site of Twist1 by mass spectrometry and with specific antibodies. This Ser 68 is phosphorylated by p38, c-Jun N-terminal kinases (JNK), and extracellular signal-regulated kinases1/2 in vitro, and its phosphorylation levels positively correlate with Twist1 protein levels in human embryonic kidney 293 and breast cancer cells. Prevention of Ser 68 phosphorylation by an alanine (A) mutation (Ser 68A) dramatically accelerates Twist1 ubiquitination and degradation. Furthermore, activation of mitogen-activated protein kinases (MAPK) by an active Ras protein or TGF-β treatment significantly increases Ser 68 phosphorylation and Twist1 protein levels without altering Twist1 mRNA expression, whereas blocking of MAPK activities by either specific inhibitors or dominant negative inhibitory mutants effectively reduces the levels of both induced and uninduced Ser 68 phosphorylation and Twist protein. Accordingly, the mammary epithelial cells expressing Twist1 exhibit much higher degrees of EMT and invasiveness on stimulation with TGF-β or the active Ras and paclitaxel resistance compared with the same cells expressing the Ser 68A-Twist1 mutant. Importantly, the levels of Ser 68 phosphorylation in the invasive human breast ductal carcinomas positively correlate with the levels of Twist1 protein and JNK activity and are significantly higher in progesterone receptor-negative and HER2-positive breast cancers. These findings suggest that activation of MAPKs by tyrosine kinase receptors and Ras signaling pathways may substantially promote breast tumor cell EMT and metastasis via phosphorylation and stabilization of Twist1. Cancer Res; 71(11): 3980–90. ©2011 AACR.

Introduction

Mitogen-activated protein kinases (MAPK), including extracellular signal-regulated kinases (ERK), c-Jun N-terminal kinases (JNK), and p38 isoforms (p38-α and -β), are serine (Ser)/threonine (Thr)-specific protein kinases. These MAPKs respond to extracellular stimuli and regulate various cellular activities (1). In human breast cancer, activation of MAPK signaling cascades increases cell proliferation, inhibits cell apoptosis, and promotes metastasis (2, 3). The increased JNK activity positively correlates with the tamoxifen resistance in breast cancer (4, 5). JNK activity is also involved in the activation of activation protein (AP)-1 and NF-κB transcription factors in glucocorticoid-induced mammary epithelial acinar formation in 3-dimensional culture (6).

The active Ras drives the activation of MAPKs in breast cancer (7–9). Overexpression of H-Ras or expression of constitutively active H-RasV12 mutant, which activates MAPKs, is fully capable of inducing metastatic mammary tumors in mice or of transforming fibroblasts into metastatic tumor-forming cells (9, 10). Expression of H-Ras also induces human MCF-10A mammary epithelial cells to form ductal carcinoma in situ, followed by development of invasive ductal carcinoma in mouse mammary glands (11). MAPKs activate a number of transcription factors important for tumor cell initiation, growth, and survival, such as the estrogen receptor (ERα), AP-1, NF-κB, and Ets family transcription factors (12–15). A recent study also showed that ERK2 is responsible for mediating Ras-induced epithelial–mesenchymal transition (EMT) in MCF-10A cells (16). However, it remains unclear how MAPKs regulate transcription factors such as Twist1 that promotes breast tumor cell EMT, invasiveness, and metastasis.

Twist1 is a member of the basic helix–loop–helix (bHLH) transcription factor family. In breast cancer, Twist1 expression is increased in patients associated with poor survival and metastasis, whereas knockdown of Twist1 reduces breast...
cancer cell invasiveness and metastasis (17). Twist1 mainly enhances cancer metastasis by repressing E-cadherin expression and promoting EMT, cell migration, and cell invasion (18–20). Our recent study reported that Twist1 interacts with several components of the NuRD complex including MTA2, RbAp46, Mi-2, and HDAC2 and recruits this complex to the E-cadherin promoter to repress E-cadherin expression and promote cancer cell EMT and metastasis. Knockdown of Twist1, MTA2 or RbAp46 in 4T1 and MDA-MB-435 cells inhibits their metastasis in vivo (21). Another study also reported that Twist1-induced EMT could generate cells that share properties with cancer stem cells (22). In addition, Twist1 can also work cooperatively with oncogenic proteins, such as Ras or ErbB2, to induce complete EMT by overriding oncogene-induced premature senescence (23).

Here, we report that Twist1 is phosphorylated at Ser 68 by Ras-activated JNK, ERK, and p38 MAPKs, and this posttranslational modification is required to maintain Twist stability and its stability-dependent functions in controlling EMT and cell invasion. Furthermore, the levels of Twist1 phosphorylation at Ser 68 in human HER2-positive ductal carcinomas correlate positively with the levels of Twist1 protein and JNK activities but negatively with progesterone receptor (PR) expression. These findings suggest that MAPK-mediated Twist1 phosphorylation and stabilization play an important role in breast cancer cell EMT and invasion.

Materials and Methods

The inducible human embryonic kidney (HEK) 293 cell lines expressing Flag (F) or F-tagged Twist1 (F-Twist1) were generated as previously described (21). Both types of cells were treated with 0.1 μg/mL of doxycyclin (DOX) for 6 hours to induce F and F-Twist1 expression. Clear cell lysates were prepared in the presence of protease inhibitor cocktail and the NaVO₃ phosphatase inhibitor and subjected to immunoprecipitation by using the anti-Flag M2 agarose beads (Sigma). After being washed thoroughly, the bound proteins were eluted by 3 × Flag peptide solution (Sigma), separated in a SDS-PAGE gel and stained with Coomassie Blue. The F-Twist1 band was excised, digested by trypsin, and analyzed by mass spectrometry to identify phosphorylation site as described previously (24).

The experimental procedures of immunoblotting, phosphorylation, protein stability, ubiquitination, reverse transcriptase (RT)-PCR, cell invasion, and human breast tumor immunostaining are described in the Supplementary Material because of the limited space.

Results

Twist1 is phosphorylated on Ser 68

To study Twist1 phosphorylation, we generated DOX-inducible 293 cell lines expressing either F or F-Twist1 and immunopurified F and F-Twist1 from these cells. Western blot analyses confirmed that F-Twist1 protein was produced in F-Twist1 293 cells but not in F 293 cells (Fig. 1A). The apparent molecular weight of F-Twist1 was slightly reduced by active λ-PPase treatment but not by heat-inactivated λ-PPase (Supplementary Fig. S1A), suggesting that F-Twist1 is a phosphorylated protein. Furthermore, F-Twist1 positively reacted with pSer antibody but not pTyr antibody, indicating that F-Twist1 contains phosphorylated serine residue(s) (Fig. 1A).

To map the phosphorylation site(s), the F-Twist1 band was excised from the gel, digested by trypsin, and subjected to mass spectrometry analysis. This unbiased approach identified only Ser 68 as the phosphorylated residue in F-Twist1 (Supplementary Fig. S2). This assay was done twice with 2 batches of purified F-Twist1; the same results were uniform across all trials. To evaluate the effects of pSer 68 on F-Twist1 molecular features, we mutated Ser 68 to alanine (Ser 68A) and glutamine (Ser 68E) and expressed these mutants in inducible 293 cells. Both mutant proteins showed slightly reduced apparent molecular weights when compared with wild-type F-Twist1 and had no detectable phosphoserine residue (Supplementary Fig. S1B). These results show that Ser 68 is the major phosphorylation site of F-Twist1 in 293 cells.

A short Twist1 peptide containing pSer 68 was used to generate rabbit antiserum. From the antiserum, the pSer 68–Twist1–specific and pSer 68-insensitive Twist antibodies were purified. As expected, the pSer 68–Twist1 antibody specifically recognized the HA-Twist1 with Ser 68 but not the HA-Twist1-Ser 68A and HA-Twist1-Ser 68E mutants, whereas pSer 68–Twist1 insensitive antibody recognized all 3 proteins (Fig. 1B1). Using these antibodies, we measured the levels of total Twist1 and pSer 68–Twist1 in several cell lines. The Twist1 level is high in MDA-MB-435 and 4T1 metastatic breast cancer cells and low in MCF-10A mammary epithelial cells, nonmetastatic Erbb2-positive MCF-7, and T47D breast cancer cells, and moderately invasive Erbb2-negative MDA-MB-231 and Sum1315 breast cancer cells. Interestingly, the pSer 68–Twist1 levels positively correlate with total Twist1 levels in these cells (Fig. 1B2). These results suggest a potential link that relates pSer 68–Twist1, Twist1 concentration, and breast cancer metastasis.

pSer 68 stabilizes Twist1 by protecting Twist1 from ubiquitination

We expressed Twist1, Twist1-Ser 68A, and Twist1-Ser 68E as GFP fusion proteins in HeLa cells and examined their subcellular localization by fluorescence microscopy. All 3 fusion proteins were observed predominantly in the nucleus (Supplementary Fig. S3A), whereas the GFP control protein was observed in both the cytoplasm and nucleus (data not shown). These results indicate that pSer 68 is not required for Twist1 nuclear localization. Next, because Twist1 forms a heterodimer with E12 (25), we compared the heterodimerization function of Twist1 to that of Twist1-Ser 68A by fusing E12 to the Gal4 DNA-binding domain, and Twist1 and Twist1-Ser 68A to the VP16 transcriptional activation domain in a mammalian 2-hybrid system by using UAS-tk-luciferase reporter. However, no difference in reporter activity was found in these assays (Supplementary Fig. S3B), suggesting that pSer 68 is not required for the Twist dimerization function. Finally, we assessed the stability of Twist1 and Twist1-Ser 68A proteins after protein synthesis was blocked in chemotherapy-treated 293 cells. The
half-lives of HA-Twist1 and HA-Twist1-Ser 68A in the transfected 293 cells were 5.8 and 1.9 hours (Fig. 1C). Similar results were obtained from inducible 293 cell lines expressing F-Twist1 and F-Twist1-Ser 68A, in which Ser 68A mutation decreased the half-life of Twist1 from 6.2 to 1.8 hours (Supplementary Fig. S1C and D).

To assess the molecular mechanism underlying pSer 68-promoted Twist1 stability, we expressed HA-ubiquitin in the inducible F-Twist1, F-Twist1-Ser 68A, and F-Twist1-Ser 68E cell lines and immunoprecipitated these F-tagged proteins for ubiquitination assay. The association of Twist1 with HA-ubiquitin appeared as typical high-molecular-weight ladders. Blocking of proteasomal activity by inhibitor MG132 resulted in slightly higher accumulations of Twist1-ubiquitin complexes, suggesting that Twist1 protein was polyubiquitinated before proteasome-mediated degradation (Fig. 1D). Importantly, coexpression of HA-ubiquitin with F-Twist1-Ser 68A or F-Twist1-Ser 68E robustly increased the degrees of ubiquitination of these proteins when compared with the F-Twist1 protein. The increase in ubiquitination of these mutant Twist1 proteins was particularly evident in the presence of MG132 (Fig. 1D). These results indicate that phosphorylation of Ser 68 protects Twist1 from ubiquitination and degradation.

Ser 68 in Twist1 is phosphorylated by MAPKs

Sequence analysis suggested that Ser 68 in Twist1 lies in a Ser/Thr-Pro consensus phosphorylation site of MAPKs (26). Therefore, we generated and purified recombinant glutathione S-transferase (GST) protein and GST fusion protein containing the N-terminal 112 a.a. (Twist1-N) and conducted in vitro phosphorylation assays with members of the MAPK family including p38, JNK, ERK1, and ERK2. The assays showed that all 4 MAPKs efficiently phosphorylated Ser 68 in Twist1-N, as detected by the pSer 68-specific antibody. The

Figure 1. Twist1 expression, purification, phosphorylation and stability assays. A, immunoprecipitated F-Twist1 (F-T) was analyzed by immunoblotting (IB) with antibodies against Flag, p-Serine (pSer), and p-Tyrosine (pTyr). Immunoprecipitation from F cells served as a negative control. IgG heavy and light chains. B1, 293 cells were transfected with the indicated plasmids. Cell lysates were assayed by IB with the indicated antibodies. HA-T, HA-tagged Twist1; p-Ser 68, pSer 68-Twist1. B2, the cell lysates were prepared from the indicated cell lines and analyzed by IB with antibodies against Twist1, pSer 68-Twist, and β-actin. C, 293 cells were transfected with HA-Twist1 or HA-Ser 68A-Twist1 plasmids. After 12 hours, cells were treated with cycloheximide (CHX) for time periods as indicated. IB was conducted with HA and tubulin antibodies. Densitometric values were determined and presented. The half-lives (50%) of HA-Twist1 and HA-Ser 68A-Twist1 are indicated (WT, wild type). D, F (−), F-Twist1 (W), F-Ser 68A-Twist1 (A), and F-Ser 68E-Twist1 (E) inducible 293 cells were transfected with mock plasmids or HA-ubiquitin expression plasmids as indicated. After 12 hours of transfection, cells were treated with DOX for 6 hours before cells were treated with a vehicle or MG132 for another 6 hours. Immunoprecipitation was carried out with Flag antibody, followed by IB with HA and Flag antibodies as indicated, ns, nonspecific band.
HA-Twist1 plasmids in combination with H-RasV12 (GST-Twist1-N proteins served as the loading control. SB203580, a p38 MAPK inhibitor; SP60125, a JNK inhibitor. B, 293 cells were transfected with mutant and had little effect on their mRNA levels (Supplementary Fig. S4). These results show that activation of the Ras pathway in cells dramatically enhances Twist1 stability through stimulating Ser 68 phosphorylation.

Ras activates multiple MAPKs (27–29). To define the Ras-activated MAPKs that phosphorylate Ser 68 of Twist1, we treated Twist1 and H-RasV12–transfected 293 cells with specific MAPK inhibitors and coexpressed specific MAPK-dominant negative inhibitory mutants (DNIM) in these cells to inhibit individual p38, JNK, and ERK kinases. SB203580 treatment slightly reduced and expression of the p38 DNIM (30) significantly reduced the active form of p38, p-p38, which was associated accordingly with a moderate and a more dramatic reduction in pSer 68-Twist1 and Twist1 proteins, respectively (Fig. 3A and B). SP60125 treatment effectively reduced p-JNK levels and also reduced pSer 68-Twist1 and total Twist1 proteins (Fig. 3A). The JNK DNIM inhibits JNK activity by competing with wild type JNK for activation and substrate binding (31). Expression of JNK DNIM efficiently decreased the levels of pSer 68-Twist1 and total Twist1, although it only partially reduced p-JNK levels (Fig. 3B). Inhibition of ERK activity by PD98059 treatment or MEK DNIM (32) expression similarly reduced pSer 68-Twist1 and Twist1 protein levels (Fig. 3A and B). Furthermore, inhibition of p38, JNK, or ERK kinases in metastatic 4T1 breast cancer cells expressing high endogenous Twist1 also decreased pSer 68-Twist and total Twist1 proteins with variable efficacies. Among them, the JNK inhibitor treatment reduced both p-JNK and p-ERK levels, and thereby was particularly potent in reducing both the pSer 68-Twist1 and total Twist1 levels (Fig. 3C). These results indicate that all 3 subfamily members of MAPKs are capable to

Figure 2. MAPKs phosphorylate Twist1 at Ser 68 in vitro and Ras activation stabilizes Twist1 in HEK293 cells. A, GST control and GST-Twist1-N proteins were incubated with MAPKs as indicated for in vitro phosphorylation assays. IB was carried out with pSer 68-Twist1 antibody. Coomassie Blue staining of GST and GST-Twist1-N proteins served as the loading control. SB203580, a p38 MAPK inhibitor; SP60125, a JNK inhibitor. B, 293 cells were transfected with HA-Twist1 plasmids in combination with H-RasV12 (+) plasmids or its mock vector (−). H-Ras, Twist1, and glyceraldehydes-3-phosphate dehydrogenase (GAPDH) mRNAs were measured by RT-PCR. H-Ras, pSer 68-Twist1, and tubulin proteins were measured by IB. C, 293 cells were transfected with H-RasV12 plasmids in combination with HA-Twist1 or HA-Ser 68A-Twist1 plasmids. After 12 hours, cells were treated with cycloheximide (CHX) as indicated. IB was carried out with antibodies against HA (for Twist1), H-Ras, and tubulin (WT, wild type). Densitometric values were determined and plotted.

GST control did not show any phosphorylation in the same assay. The p38- and JNK-mediated Ser 68 phosphorylations were blocked by their respective specific kinase inhibitors, SB203580 and SP60125 (Fig. 2A). Thus, the Ser 68 in Twist1 is an ideal target site for MAPKs.

We further investigated the signaling pathway that regulates pSer 68 in Twist1. It has been shown that Twist1-promoted EMT can be further enhanced by Ras or ErbB2 oncoproteins (23). To investigate whether the Ras-activated MAPKs phosphorylate Twist1 on Ser 68 in vivo, we coexpressed HA-Twist1 with either H-RasV12, a constitutively active form of human H-Ras, or a control plasmid in 293 cells. The Twist1 mRNA levels were comparable across cells with and without H-RasV12 expression. However, H-RasV12 expression robustly increased the level of pSer 68, which was accompanied by increased total Twist1 protein (Fig. 2B). To determine whether H-RasV12–stimulated pSer 68 increases the stability of Twist1, we coexpressed either Twist1 or Twist1-Ser 68A with H-RasV12 and carried out a CHX-based protein chase experiment. H-RasV12 expression extended the half-life of Twist1 from 5.8 to 9.8 hours, whereas it did not significantly change the half-life of Twist1-Ser 68A, which was 1.9 hours in both the presence and absence of H-RasV12 (compare Fig. 2C with Fig. 1C). To extrapolate our observations to breast epithelial cells, we generated a series of MCF-10A stable cell lines by using retroviruses that mediate the expression of Twist1, Twist1-Ser 68A, and H-RasV12 in combination. Again, H-RasV12 selectively increased the Twist1 level in MCF-10A cells without stabilizing the Twist1-Ser 68A mutant and had little effect on their mRNA levels (Supplementary Fig. S4). These results show that activation of the Ras pathway in cells dramatically enhances Twist1 stability through stimulating Ser 68 phosphorylation.

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phosphorylate and stabilize Twist1 protein in both 293 and breast tumor cells.

Ser 68 phosphorylation is required for Twist1-promoted EMT and breast cancer cell invasion

To understand the role of pSer 68 in Twist1 function, we first examined how Twist1 phosphorylation contributed to Twist1-promoted EMT. Among 3 stable MCF-10A human mammary epithelial cell lines expressing F tag (control), F-Twist1, or F-Twist1-Ser 68A, F-Twist1 protein was steadily detected, whereas Twist1-Ser 68A protein was barely detected because of its instability, even though both cell lines expressed comparable Twist1 and Twist1-Ser 68A mRNAs (Fig. 4A). As expected from previous studies (17), Twist1 expression inhibited the expression of epithelial markers including E-cadherin, β-catenin, and γ-catenin, but promoted the expression of mesenchymal marker vimentin in MCF-10A cells. However, Twist1-Ser 68A expression failed to inhibit these epithelial markers and only slightly promoted vimentin expression (Fig. 4A). Similar results were also obtained from E-cadherin immunostaining, showing higher E-cadherin signals in Twist-Ser 68A cells versus Twist1 cells (Fig. 4B). These results suggest that Ser 68 phosphorylation is required for Twist1-promoted EMT.

Ras can transform MCF-10A cells (33) and work with Twist1 in MDCK cells to achieve a complete EMT phenotype (23). To examine the specific role of pSer 68 as an effector in the Ras signaling, we generated MCF-10A cell lines expressing H-RasV12 and either Twist1 or Twist1-Ser 68A. Either H-RasV12 expression alone or accompanied by Twist1 or Twist1-Ser 68A diminished E-cadherin expression, suggesting that H-RasV12 alone is sufficient to induce EMT in MCF-10A cells under our experimental conditions (data not shown). However, cells expressing H-RasV12 and Twist1 showed morphology closer to that of fibroblasts (Fig. 4C). More importantly, MCF-10A cells expressing Twist1 or Twist1-Ser 68A alone showed little change in cell invasion through a Matrigel layer when compared with control MCF-10A cells, whereas coexpression of Twist1 and H-RasV12 robustly increased cell invasiveness. However, expression of H-RasV12 alone only slightly increased cell invasiveness, and coexpression of Twist-Ser 68A and H-RasV12 only moderately increased cell invasiveness (Fig. 4C). These results show that Ser 68 phosphorylation is required for Ras-stimulated and Twist1-promoted cell invasiveness.

TGF-β activates Ras/MAPK pathways such as p38 MAPK and regulates gene expression to promote EMT and cancer metastasis (34–36). Thus, we examined whether TGF-β could induce Twist1 phosphorylation on Ser 68 and twist protein, suggesting that H-RasV12 alone is sufficient to induce EMT in MCF-10A cells under our experimental conditions (data not shown). However, cells expressing H-RasV12 and Twist1 showed morphology closer to that of fibroblasts (Fig. 4C). More importantly, MCF-10A cells expressing Twist1 or Twist1-Ser 68A alone showed little change in cell invasion through a Matrigel layer when compared with control MCF-10A cells, whereas coexpression of Twist1 and H-RasV12 robustly increased cell invasiveness. However, expression of H-RasV12 alone only slightly increased cell invasiveness, and coexpression of Twist-Ser 68A and H-RasV12 only moderately increased cell invasiveness (Fig. 4C). These results show that Ser 68 phosphorylation is required for Ras-stimulated and Twist1-promoted cell invasiveness.
stabilize Twist1, we treated MCF-10A cell lines expressing either Twist1 or Twist1-Ser 68A with TGF-β. TGF-β treatment significantly increased pSer 68-Twist1 and total Twist1 protein levels in Twist1-expressing cells, but failed to increase the Twist1-Ser 68A protein in Twist1-Ser 68A-expressing cells. In fact, Twist1-Ser 68A was difficult to detect because of its instability, regardless of TGF-β treatment (Fig. 5C). These results indicate that activation of the TGF-β/MAPK signaling pathways stabilizes Twist1 protein through induction of Twist1 phosphorylation on Ser 68.

To estimate the role of Ser 68 phosphorylation in TGF-β–induced cell invasion, we conducted cell invasion assays in the presence or absence of TGF-β with MCF-10A cell lines expressing Twist1, Twist1-Ser 68A or mock control. The vehicle-treated mock, Twist1, and Twist1-Ser 68A–expressing cells and TGF-β–treated mock and Twist1-Ser 68A–expressing cells showed discernible, but insignificant, changes in their invasion capabilities. However, TGF-β–treated Twist1-expressing cells showed a robust increase in cell invasion capability (Fig. 5C). These results suggest that Ser 68 phosphorylation plays an important role in mediating TGF-β–induced and Twist1-promoted cell invasion.

Twist1 is known to promote cell survival and increases cellular resistance to paclitaxel-induced apoptosis (37). On paclitaxel treatment, the MCF-10A cells expressing Twist1-Ser 68A exhibited a slightly increased viability than MCF-10A cells with the mock vector, but a remarkably decreased viability than MCF-10A cells expressing wild-type Twist1 (Supplementary Fig. S5). These results suggest that prevention of Twist1 from Ser 68 phosphorylation reduces its function to enhance paclitaxel resistance.

The Twist1 levels in invasive breast ductal carcinomas correlate positively with p68-Twist1 and active p-JNK and negatively with PR levels

To explore the clinical implications of pSer 68-Twist1, we investigated the correlations among pSer 68-Twist1, Twist1, and active forms of MAPKs in 24 invasive human breast tumor samples by Western blot (Fig. 6A). Among these tumor samples, levels of pSer 68-Twist1 and Twist1 proteins were found to be closely associated (Fig. 6B), supporting the finding that phosphorylation of Ser 68 stabilizes Twist1 protein not only in breast cancer cell lines but also in human breast tumors. Both pSer 68-Twist1 and Twist1 levels correlated positively and closely with active p-JNK levels (Fig. 6C). However, levels of pSer 68-Twist1 and Twist1 proteins showed no obvious correlations with active forms of ERK1/2 and p38 MAPKs (Fig. 6A). These results suggest that JNK may play a role more dominant than other MAPKs in phosphorylating Ser 68 of Twist1 in invasive breast cancers.

Among the 24 tumors, there were 11 ERα+/PR–;HER2+ tumors in group A, 5 ERα+/PR–;HER2+ tumors in group B, and 8 ERα–;PR–;HER2+ tumors in group C. According to clinical diagnosis, 22 tumors were invasive ductal carcinomas, and the remaining 2 were an invasive lobular adenocarcinoma in group A (#a4) and a lipid-rich epithelial carcinoma in group C (#e8; Fig. 6A). High-level pSer 68-Twist1 and Twist1 proteins (band intensity > 10) were detected in 2 of 11 (18%) tumors in group A, all tumors in group B, and 6 of 8 (75%) tumors in group C (Fig. 6A). The average abundances of pSer 68-Twist1 and Twist1 in groups B and C were significantly higher than that of group A (Fig. 6D). Similarly, high-level p-JNK (band intensity > 34) was found in 1 of 11 (9%) tumors in group A, 4

**Figure 4.** Ser 68 phosphorylation is required for Ras-stimulated and Twist1-promoted breast cancer cell invasion. A, stable MCF-10A cell lines with mock control, Twist1 expression, or Twist1-Ser 68A expression were assayed by IB with antibodies indicated. Twist1, Ser 68A-Twist1, and GAPDH mRNAs were measured by RT-PCR. B, the above cells were immunostained by the E-cadherin (E-Cad.) antibody (green color). Cell nuclei were stained by DAPI (right). C, morphologies of MCF-10A cell lines expressing H-RasV12 and Twist1, Ser 68A-Twist1, or a mock control as indicated (top). Real-time cell invasion assays (bottom) were conducted with the 3 cell lines in the top and the 3 cell lines in A (WT, wild type).
of 5 (80%) tumors in group B, and all 8 tumors in group C (Fig. 6A). The average levels of p-JNK in tumors of groups B and C were also significantly higher than that of the group A tumors (Fig. 6D).

Tumors with high levels of pSer 68-Twist1, Twist1, and p-JNK also exhibited higher rates of cell proliferation as detected by Ki67 immunostaining (Fig. 6A). Among the 22 tumors stained by Ki67 antibody, the 12 tumors with low pSer 68-Twist1 or Twist1 (either band intensity < 10; Fig. 6A) showed an average of 10.6 ± 8.5 (%) Ki67-positive cells, whereas the 10 tumors with high pSer 68-Twist1 and Twist1 (both band intensities > 10; Fig. 6A) showed a significantly higher average of 40.5 ± 22 (%) Ki67-positive cells (P < 0.001 by unpaired t test).

Discussion

Similar to other signaling proteins, transcription factors are commonly regulated by phosphorylation in response to various cellular signals. The transcriptional activities of several bHLH transcription factors, such as E47, Id3, and Twist1, are modulated by phosphorylation (38–40). Twist1 dimerization and DNA binding are also enhanced by PKA-mediated phosphorylation at Thr 125 and Ser 127 but inhibited by PP2A-mediated dephosphorylation of these residues (40, 41). Furthermore, the phosphorylation-regulated dimerization of Twist1 and Hoxd2 plays an essential role in limb development in both mice and chicks (42). In addition, Twist1 is phosphorylated at Ser 42 by Akt, and this modification is required for Twist1 to inhibit p53-mediated cell apoptosis in response to DNA damage (43).

In this study, we found that Ser 68 is a major phosphorylation site in Twist1 expressed in 293 cells; it was the only site detected by the mass spectrometry, and the single Ser 68A mutation diminished overall serine phosphorylation in the entire Twist1 protein. Our data showed that Ser 68
phosphorylation is not required for Twist1 nuclear localization and dimerization, indicating that Ser 68 phosphorylation has a function distinct from Thr 125 and Ser 127 phosphorylation. However, the relationship between Ser 68 phosphorylation and Ser 42 phosphorylation in p53 function is currently unknown.

MAPK-mediated phosphorylation plays crucial roles in stabilizing important transcription factors. For example, JNK-mediated phosphorylation increases p53 stability and transcriptional activity in response to stress (44). The Ras-ERK MAPK cascade protects GATA3 in Th2 cell differentiation and Myc in cell growth and oncogenesis from degradation by the ubiquitin-proteasome pathway (45, 46). Considering the importance of Twist1 in development and cancer, the cellular amount and function of Twist1 must be strictly controlled. It has been shown that a truncated Twist1 mutant associated with Saethre-Chotzen syndrome is unstable (25), and that Twist1-mediated inhibition of bone morphogenetic protein signaling is counter-regulated by E47 interaction-enhanced and Id1 interaction-
reduced Twist1 stability (47). In this study, we have shown that Ser 68A mutation significantly decreases Twist1 stability in both transiently transfected cells and stable cell lines. On the basis of a previous study showing Twist1 degradation by the ubiquitination-proteasome pathway (48), this study further showed that prevention of Ser 68 phosphorylation in Twist1-Ser 68A accelerates Twist1 polyubiquitination, which is consistent with its rapid degradation. However, the Twist1-Ser 68E mutation failed to mimic the role of Ser 68 phosphorylation. Although the reason is unclear, phosphoserine residue has 2 units of negative charges but the E residue has only 1, and the side chain of phosphoserine is also more bulky than the E side chain. These differences may allow phosphoserine and E to have different capabilities for protein–protein interaction, and thereby different stabilities.

Furthermore, we have provided compelling evidence that Twist1 is phosphorylated at Ser 68 by MAPKs. Interestingly, the Ser 68 can be phosphorylated by all p38, JNK, and ERK1/2 MAPKs in vitro and in 293 cells expressing an active H-RasV12. In 4T1 cells, all inhibitors for p38, JNK, or ERK reduced Twist1 phosphorylation and protein levels. Among the used inhibitors, the SP60125 treatment caused reduction of both active forms of p-JNK and p-ERK, resulting in additive suppression of Twist1 phosphorylation and total protein levels. These results indicate that MAPK-mediated Ser 68 phosphorylation inhibits Twist1 degradation by the ubiquitin-proteasome pathway. These findings make Twist1 a direct MAPK target important for EMT and metastasis. However, although all 3 subfamily members of MAPKs can phosphorylate Twist1 at Ser 68, the specific MAPK that ultimately plays the dominant role may depend on cellular context.

Twist1 accumulation caused by Ras/MAPK signaling more effectively enhances Twist1-mediated EMT and cell invasion, as shown by the more obvious changes in EMT markers in Twist1-expressing MCF-10A cells as compared with Twist1-Ser 68A–expressing cells. TGF-β activates p38 MAPK and induces EMT and invasiveness in breast tumor cells. In our experiment, TGF-β predominantly activated p38 MAPK in human breast cancer cells, resulting in a clear increase in Twist1 protein that was sensitive to the inhibitor of p38 MAPK. Moreover, the Twist1-promoted MCF-10A cell invasion was tremendously enhanced by both active Ras and TGF-β stimulation when compared with Twist1-Ser 68A–expressing MCF-10A cells that received the same treatments. These results suggest that MAPK-mediated Twist phosphorylation at Ser 68 may carry out an important function in the promotion of breast cancer cell EMT and invasion. Taken together, our findings are summarized in Fig. 5D.

A recent study has shown that Ras/ERK2 signaling upregulates Fra1 to induce ZEB1/2 expression, which subsequently promotes cell EMT, migration, and invasion (16). This pathway runs parallel to the pathway defined in this study, which shows that Ras-activated MAPKs phosphorylate and stabilize Twist1 to promote breast tumor cell EMT and invasion. Thus, multiple downstream molecular effectors are responsible for mediating the effects of MAPKs on breast tumor cell EMT, migration, invasion, and metastasis. These findings may explain why super activation of Ras can induce a full EMT phenotype whereas overexpression of Twist1 induces only a partial EMT phenotype in noncancerous mammary epithelial cells such as MCF-10A and epithelial-like/nonmetastatic breast cancer cells such as MCF-7 cells (data not shown).

In HER2-positive human breast tumors, we found that the levels of Twist1 protein positively correlate with levels of Ser 68 phosphorylation and active JNK, but not with those of active ERK1/2 and p38 MAPKs. These observations suggest that JNK activation could be the primary MAPK in stabilizing Twist1 in these tumors. In Drosophila, JNK controls border cell cluster integrity and collective cell migration and also induces pseudo-EMT and cell mobility during imaginal disc eversion (49, 50). In mouse keratinocytes, JNK can mediate TGF-β–induced EMT (51). In human MCF-7 breast cancer cells, TNFα can induce JNK and cell invasiveness, whereas it does not do so in immortalized mammary epithelial cells (52). Taking into account our data showing that JNK phosphorylates Twist1 in vitro and in multiple cell lines, and that Twist1 phosphorylation and protein levels correlate positively with JNK activity in invasive breast ductal carcinomas, we propose a molecular pathway that connects active JNK to Twist1 phosphorylation and accumulation, and to Twist1-promoted EMT and metastasis in human breast cancer.

Finally, our data show that high levels of total Twist1, phosphorylated Twist1, and active JNK are associated with PR-negative status in breast tumors with or without ERα. It has been shown that PR-negative status is associated with loss of responsiveness to endocrine therapy (53) and that the PR−/ER− breast cancer is associated with bone metastasis (54). Our data therefore suggest a possible link between Twist1 elevation and endocrine therapy resistance and bone metastasis. These findings also suggest that JNK and Twist1 may be potential therapeutic targets in ERα+/PR− and ERα−/PR− breast tumors.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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

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