Cell

P68 RNA Helicase Mediates PDGF-Induced Epithelial Mesenchymal Transition by Displacing Axin from β-Catenin

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SUMMARY

The nuclear p68 RNA helicase (referred to as p68) is a prototypical member of the DEAD box family of RNA helicases. The protein plays a very important role in early organ development. In the present study, we characterized the tyrosine phosphorylation of p68 under platelet-derived growth factor (PDGF) stimulation. We demonstrated that tyrosine phosphorylation of p68 at Y593 mediated PDGF-stimulated epithelial-mesenchymal transition (EMT). We showed that PDGF treatment led to phosphorylation of p68 at Y593 in the cell nucleus. The Y593-phosphorylated p68 (referred to as phosphor-p68) promotes β-catenin nuclear translocation via a Wnt-independent pathway. The phosphor-p68 facilitates β-catenin nuclear translocation by blocking phosphorylation of β-catenin by GSK-3β and displacing Axin from β-catenin. The β-catenin nuclear translocation and subsequent interaction with the LEF/TCF was required for the EMT process. These data demonstrated a novel mechanism of phosphor-p68 in mediating the growth factor-induced EMT and uncovered a new pathway to promote β-catenin nuclear translocation.

INTRODUCTION

Epithelial-mesenchymal transition (EMT) is a process by which epithelial cells undergo dramatic morphology changes marked by loss of their polarity and cell-cell contacts (Savagner, 2001). The molecular hallmarks for EMT are downregulation of epithelial cell adherens molecules, such as E-cadherin, α-catenin, and γ-catenin, and upregulation of molecules of mesenchymal components, such as vimentin, fibronectin, and smooth muscle actin (sm-actin) (Yang et al., 2004a). The EMT process is vital for morphogenesis during embryonic development and tissue remodeling (Kalluri and Neilson, 2003; Savagner, 2001). During tumor progression, substantial evidences suggest that EMT plays a critical role for tumor cells to leave an organized epithelial layer and gain the property of invasiveness. Such changes usually initiate cancer metastasis (Kang and Massague, 2004; Thiery and Chopin, 1999).

A number of cell signaling pathways stimulate EMT, such as epidermal growth factor (EGF), transforming growth-factor-β (TGF-β), hepatocyte growth factor (HGF), and platelet-derived growth factor (PDGF) (Grande et al., 2002; Lu et al., 2001; Lu et al., 2003; Nawshad et al., 2005; Pagan et al., 1999). Many of these growth factors act through their cell-surface receptors, receptor tyrosine kinases (RTKs) (Blume-Jensen and Hunter, 2001). These RTKs activate a series of downstream targets. For instance, the canonical Ras pathway (Boyer et al., 1997) and Src family of protein tyrosine kinases (Calautti et al., 1998) play critical role(s) in induction of EMT under growth-factor stimulation. Nuclear import of β-catenin is another important player in EMT (Brabletz et al., 2005). β-catenin undergoes nuclear translocation under stimulations of Wnt signal and several other growth factors. The nuclear β-catenin facilitates the transcription activation by complex with transcriptional factor LEF/TCF (Nelson and Nusse, 2004). Although the detailed mechanism by which the β-catenin nuclear translocation facilitates EMT is not well understood, it is believed that regulation of components in cell adhesion and migration by complex with LEF/TCF is important (Kim et al., 2002).

Evidence suggests that c-Abl plays a role downstream of multiple growth-factor stimulations (Pendergast, 2002). PDGF stimulation activates the membrane pool of c-Abl mediated by Src family of kinases in fibroblast cells. The c-Abl kinase is a proto-oncogene nonreceptor tyrosine kinase. The kinase localizes to the plasma membrane, the cytoplasm, and the cell nucleus (Zhu and Wang, 2004). The activated c-Abl kinase targets a variety of cellular proteins, including c-jun (Barila et al., 2000), Mdm2 (Goldberg et al., 2002), and Dok1 (Woodring et al., 2004). It was shown that the nuclear pool of c-Abl played a role in response to DNA damage (Kharbanda et al., 1997; Rich et al., 2000). On the other hand, the activation of the cytoplasmic pool of c-Abl leads to changes in cell morphology in fibroblast cells (Plattner et al., 1999).
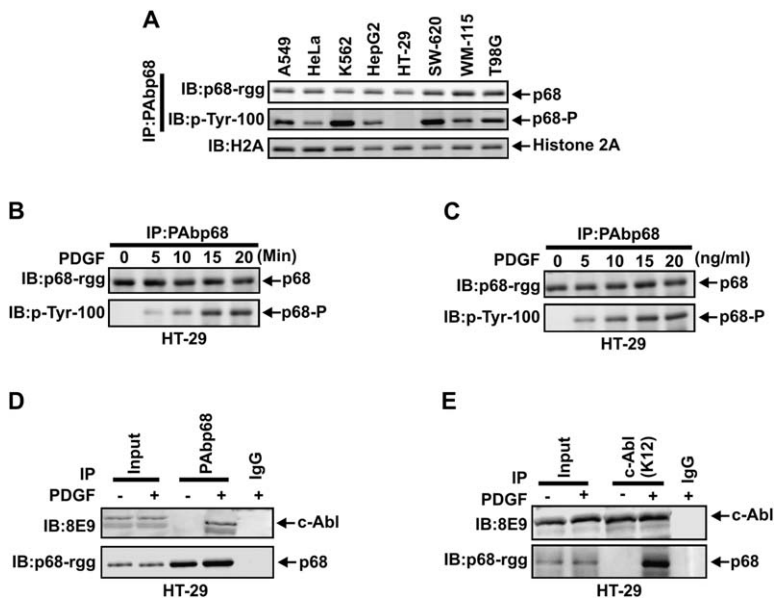
Figure 1. P68 Is Tyrosyl Phosphorylated in HT-29 Cells upon PDGF Stimulation
(A) Tyrosine phosphorylation of p68 in eight cancer cell lines (indicated). The protein was immunoprecipitated (IP) from nuclear extracts and followed by immunoblot (IB) with appropriate antibody (indicated). The IB:p68-rgg and IB:H2A were the loading controls.
(B and C) Tyrosine phosphorylation of p68 in HT-29 cells upon treatment with PDGF-BB at different time points (indicated) (B) or different doses (indicated) (C). The protein was IPed (IP:PAbp68) from nuclear extracts and IB (IB) with appropriate antibody (indicated). The IB:p68-rgg was the loading control.
(D and E) The interaction of p68 with c-Abl was detected by coIPs of p68 using antibody (PAbp68) (D) or by coIP of c-Abl using antibody against c-Abl (c-Abl[k12]) (E) followed by IBs with appropriate antibody (indicated). The IPs were carried out with nuclear extracts made from the HT-29 cells that were treated/untreated (+/−) with PDGF. Inputs were IB the extracts without IP (positive controls). Immunoprecipitation with mouse IgG was the negative control.

The nuclear p68 RNA helicase is a prototypical member of the DEAD box family of RNA helicases (Lane and Hoef-fler, 1980). As an early example of a cellular RNA helicase, the ATPase, and the RNA unwinding activities of p68 were documented (Hirling et al., 1989; Iggo and Lane, 1989). P68 plays a very important role in cell proliferation and early organ development (Stevenson et al., 1998). The protein may potentially have a role in the tumorigenesis process (Causevic et al., 2001; Wei and Hu, 2001). We have previously reported that p68 is phosphorylated at multiple amino acid residues, including serine/threonine and tyrosine (Yang et al., 2005; Yang and Liu, 2004). In the present study, we characterized the tyrosine phosphorylation of p68 stimulated by PDGF-BB treatments and found that tyrosine phosphorylation of p68 at Y593 is necessary for PDGF-stimulated EMT. We discovered that PDGF treatment activated the c-Abl kinase, which subsequently phosphorylated p68 at Y593 in the nucleus. The phosphor-p68 promoted cytoplasmic β-catenin nuclear translocation via a Wnt-independent pathway. The phosphor-p68 interacted with nuclear β-catenin, which subsequently stimulated EMT.

RESULTS

PDGF Activates c-Abl, which Subsequently Phosphorylates P68 at Y593

Our previous experiments demonstrated that p68 was phosphorylated at tyrosine residues in cancerous cell lines and the protein phosphorylation could be induced by PDGF treatment in noncancerous cells (Yang et al., 2005; Yang and Liu, 2004), suggesting that p68 may be a downstream effecter of PDGF signaling. To understand the role of the phosphor-p68 in mediating the effects of PDGF signaling, we sought to identify the protein kinase that phosphorylated p68 and to determine the phosphorylation site(s). To search for a suitable study cell system, we probed the phosphorylation status of p68 in several human cancer cell lines. Consistent with our previous observations, p68 was phosphorylated at tyrosine residue in the cancer cells, with the exception of HT-29, an epithelial colon cancer cell line (Figure 1A). We then probed whether p68 could be phosphorylated in HT-29 cells upon PDGF treatment. P68 became rapidly phosphorylated at tyrosine residue(s) in a time- and dose-dependent manner in HT-29 cells (Figures 1B and 1C). We also found that c-Abl kinase was activated upon PDGF stimulation, as demonstrated by phosphorylation of c-Abl at Y245, and the phosphorylation of c-Jun, an authentic substrate of c-Abl (Figure 2B). Moreover, the timing of the c-Abl activation correlated closely with p68 phosphorylation (Figure 2B, data not shown, also see Figure 5B). Thus, we speculate that p68 is a cellular target of c-Abl.

To test the possibility, we examined whether p68 interacted with c-Abl under PDGF treatment. It was evident that c-Abl coimmunoprecipitated with both endogenous and exogenously expressed p68. The interaction between p68 and c-Abl was dependent upon PDGF-BB treatment (Figures 1D, 1E, S1A, and S1B). We next investigated whether c-Abl phosphorylated p68. First, a bacterially expressed his-tagged p68 was phosphorylated by a recombinant c-Abl. Under the same condition, two control proteins were not phosphorylated. The phosphorylation of the his-p68 by the recombinant c-Abl was inhibited by STI571, an Abl-specific inhibitor (Figure 2A). Second, the recombinant his-p68 was phosphorylated by a complex that was pulled down from nuclear extracts made from PDGF-treated cells by using an antibody against c-Abl
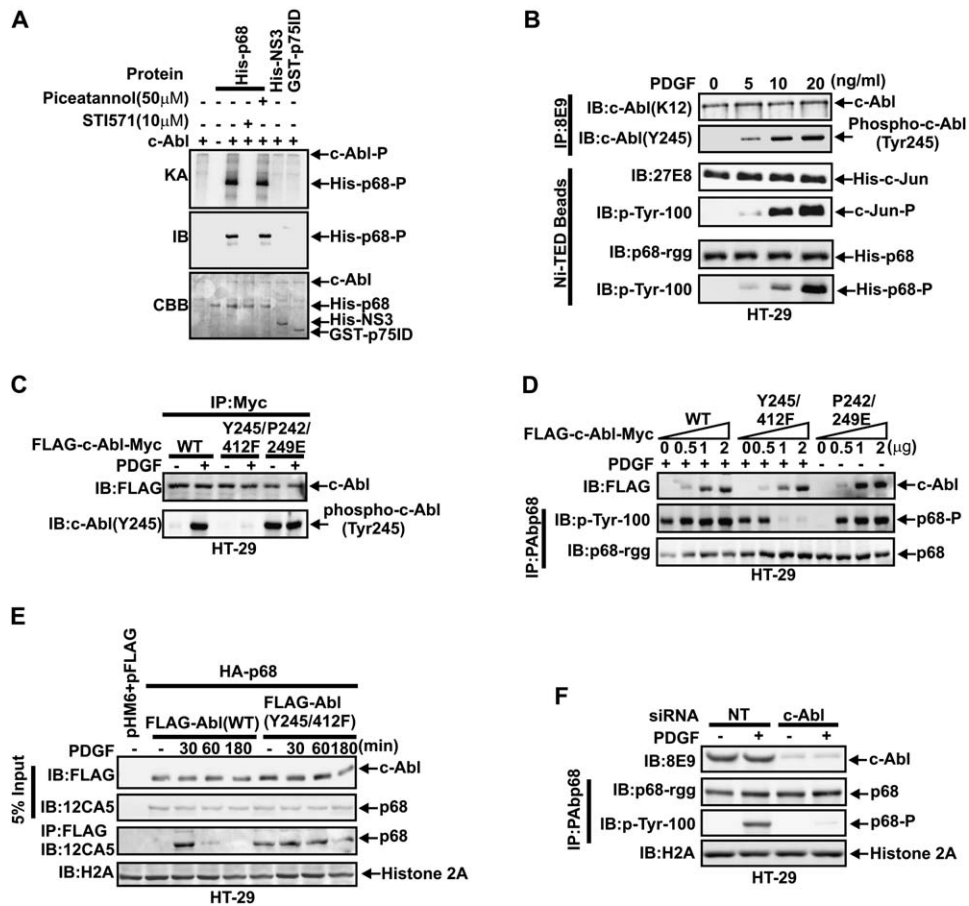
Figure 2. Phosphorylation of P68 by c-Abl

(A) In vitro phosphorylation of his-p68 by recombinant c-Abl were detected by the autoradiography (KA), immunoblotting with p-Tyr-100 (IB), and Coomassie blue staining (CBB) of the SDS-PAGE of each in vitro kinase reaction. his-NS3 and GST-p75ID were two negative control proteins for the phosphorylation. Piceatannol is a kinase inhibitor that does not target c-Abl.

(B) Phosphorylation of his-c-Jun or his-p68 by IP complex using antibody against c-Abl from nuclear extracts made from HT-29 cells that were treated with different doses of PDGF-BB (indicated). c-Abl and phosphorylated c-Abl in the IP complex (IP:8E9) was examined by IBs with appropriate antibody (indicated). The his-c-Jun or his-p68 was precipitated by Ni-TED beads and immunoblotted by appropriate antibodies (indicated) after phosphorylation by the IP complex.

(C) The Y245 phosphorylation of Flag-c-Abl-Myc (WT, Y245/412F, and P242/249E) in HT-29 cells with and without (+/−) PDGF-BB treatment. The Flag-c-Abl-Myc was precipitated by anti-Myc antibody (IP:Myc) followed by IB using antibody c-Abl (Y245) (IB:c-Abl[Y245[). The IB:FLAG was the loading control.

(D) Tyrosine phosphorylation of endogenous p68 in HT-29 cells with and without (+/−) PDGF treatment in the presence of Flag-c-Abl-Myc (WT, Y245/412F, or P242/249E) were detected by IP of p68 (IP:PAbp68) followed by IBs via antibody p-Tyr-100 (IB:p-Tyr-100). The IB:p68-rgg was the loading controls. Expression levels of Flag-c-Abl-Myc were indicated by IBs via anti-Flag antibody (IB:FLAG).

(E) CoIPs of HA-p68 with FLAG-Abl (IP:FLAG), WT or Y245/412F, at different times after PDGF stimulation (indicated) were detected by IB using antibody 12CA5 (IB:12CA5). The inputs were the IB of 5% extracts by appropriate antibodies (indicated) without IP. IB:H2A was a loading control. The pHM6 and pFLAG were the transfection of vectors.

(F) Tyrosine phosphorylation of p68 in HT-29 cells with and without (+/−) PDGF treatment was detected by IB using antibody p-Tyr-100 (IB:p-Tyr-100). P68 was immunoprecipitated by antibody PAbp68 (IP:PAbp68) from the nuclear extracts. The cells were treated with nontargeting siRNA (NT) or c-Abl siRNA (c-Abl). Knockdown of c-Abl was indicated by IB using antibody 8E9 (IB:8E9). The IB by p68-rgg is a positive control for the p68 precipitation. The IB:H2A was a loading control.

(Figure 2B). These experiments suggested that p68 can be phosphorylated by nuclear c-Abl. To further confirm the phosphorylation of p68 by c-Abl, we examined tyrosine phosphorylation of endogenously or exogenously expressed p68 in HT-29 cells, in which c-Abl wild-type (WT) or mutants (inactive mutant Y245/412F, constitu-

tively active mutant P242/249E) (Furstoss et al., 2002; Nagar et al., 2003) were expressed. PDGF treatment activated c-Abl as indicated by phosphorylation at Y245 in WT and P242/249E (Figure 2C). The endogenous p68 was phosphorylated under PDGF treatment. However, when increased amounts of inactive mutant Y245/412F

were expressed in the cells, tyrosine phosphorylation of p68 declined and almost disappeared (Figure 2D). The inactive c-Abl mutant prevented p68 from phosphorylation by persistent interaction with p68 as revealed by coimmunoprecipitation experiments (Figure 2E). Expression of constitutively active mutant P242/249E led to tyrosine phosphorylation of p68 independent of PDGF (Figure 2D). Moreover, we examined tyrosine phosphorylation of p68 in HT-29 cells, in which c-Abl was knocked down (over 90%) by RNA interference (RNAi). Under these conditions, tyrosine phosphorylation of p68 was not detected (Figure 2F). These results suggested that p68 was phosphorylated by c-Abl in HT-29 cells under stimulation of PDGF.

We next employed MALDI mass spectroscopy (MS) to identify the phosphorylation site(s). The cellular p68 was immunopurified from nuclear extracts made from PDGF-treated HT-29 cells. The purified p68 was cleaved by CNBr. MS spectrum revealed an m/z 1599.58 Da peak that corresponded to an 80 Da shift of the m/z 1519.60 Da cleavage fragment (Figures 3A and 3B). The m/z 1599.58 peak can be purified by a Pi3 PhosphoTyrosine column. Further, this shift is sensitive to tyrosine phosphatase 1B (PTP) (Figure 3C), indicating that the shift is indeed due to tyrosine phosphorylation. To further confirm the tyrosine phosphorylation, we created two mutants, Y593F and Y567F. The bacterially expressed his-p68s (WT and the mutants) were subjected to phosphorylation by the c-Abl. Both WT and mutant Y567F could be phosphorylated. In contrast, the mutant Y593F could not be phosphorylated (Figure 3D). We further tested the phosphorylation of p68 in HT-29 cells. Under stimulation of PDGF, WT p68, and mutant Y567F were phosphorylated (Figure S1C). The Y593F mutant could not be phosphorylated by c-Abl. The protein still interacted with c-Abl as revealed by coimunoprecipitation of c-Abl with Y593F (Figure S1C). There are two tyrosine residues in the 1519.60 Da fragment (Y593, Y595). To determine the phosphorylation residue, we created two mutants, Y593F and Y595F. The Y595F was tyrosyl phosphorylated in HT-29 cells under PDGF stimulation, but the Y593F was not (Figure 3E), suggesting that Y593 was the phosphorylation site.

Y593 Phosphorylation of P68 Promotes EMT
To elucidate the functions of the phosphor-p68, we first examined the morphology changes of cultured cells under stimulation of PDGF. The HT-29 cells maintain very strong cell-cell adhesion under normal culture conditions. The cells display a typical round cobble-stone shape. Treatment of HT-29 cells with PDGF-BB led to loss of cell-cell contacts and cell scattering. The cell shape also underwent dramatic changes. The cells showed more of a fibroblastic morphology (Figure 4A). This phenomenon is indicative of EMT. We then examined the effects of p68 phosphorylation at Y593 on these morphology changes. First, p68 was knocked down more than 90% by RNAi (Figure 4B). Knockdown of p68 abolished the effects of PDGF on cell morphology changes (Figure 4A). We then

expressed p68 or mutants in the p68 knockdown cells (Figure 4B). Expression of WT p68 restored the effects of PDGF on the cell morphology changes. However, expression of the Y593F mutant did not restore the effects (Figure 4A). These results indicated that phosphorylation of p68 at Y593 was required for PDGF-stimulated EMT. To further verify the functional role of the Y593 phosphorylation in EMT, we probed the changes of molecular markers that typically reflect EMT. The epithelial markers, E-cadherin, α-catenin, and γ-catenin, decreased dramatically upon PDGF treatment, while the level of mesenchymal markers, fibronectin, sm-actin, and N-cadherin, increased significantly. Knockdown of p68 abolished the effects of PDGF on the cellular levels of these markers. Expression of WT p68 after knockdown of p68 restored the effects of PDGF, especially the cellular level of sm-actin, which was several-fold higher than those in cells without p68 knockdown. Expression of p68 mutant Y593F did not restore the effects of PDGF (Figure 4C). Since p68 is phosphorylated by c-Abl, we tested whether c-Abl is required for PDGF-induced EMT. The changes in both cell morphology and molecular markers for EMT in c-Abl knockdown cells indicated that c-Abl was required for the PDGF-induced EMT (Figure 4D). Hence, changes in both cell morphology and molecular markers indicated that PDGF promoted EMT in HT-29 cells. The phosphorylation of p68 at Y593 by c-Abl is required for the PDGF-induced EMT. A very similar phenomenon was also observed with another epithelial colon cancer cell line, HCT116 (data not shown). To determine whether our observation is a general phenomenon, we examined the effects of the p68 phosphorylation on EMT in the MDCK cell system. Exogenously expressed p68 was tyrosine phosphorylated in MDCK cells upon EGF or TGF-β treatment (Figure S2A). Expression of p68 enhanced the EGF- or TGF-β-stimulated EMT in MDCK cells (Figure S2B). Knockdown of p68 largely blocked TGF-β-stimulated EMT as revealed by the changes in morphology (Figure S2C) and molecular markers of EMT (Figure S2D).

To determine whether the p68 phosphorylation is sufficient to promote EMT, we delivered in vitro phosphorylated (via c-Abl) his-p68 to HT-29 cells. Most of the his-p68 delivered to HT-29 cells localized to the cell nucleus (Figure S3A). Although, one should be cautious in interpreting the experimental results of direct delivery of a regulatory protein, it was clear that the in vitro phosphorylated p68 promoted EMT as revealed by changes in morphology and cellular level of the EMT markers (Figure 4E).

The Phosphor-P68 Interacts with Nuclear β-Catenin and Promotes the Translocation of β-Catenin to Cell Nucleus via a Wnt-Independent Pathway
To understand the mechanism by which the phosphor-p68 promotes EMT, we attempted to probe cellular proteins that might interact with the phosphor-p68. Among a number of potential candidates, β-catenin stood out due to the important role of nuclear β-catenin in mediating both cell proliferation and cell morphology change
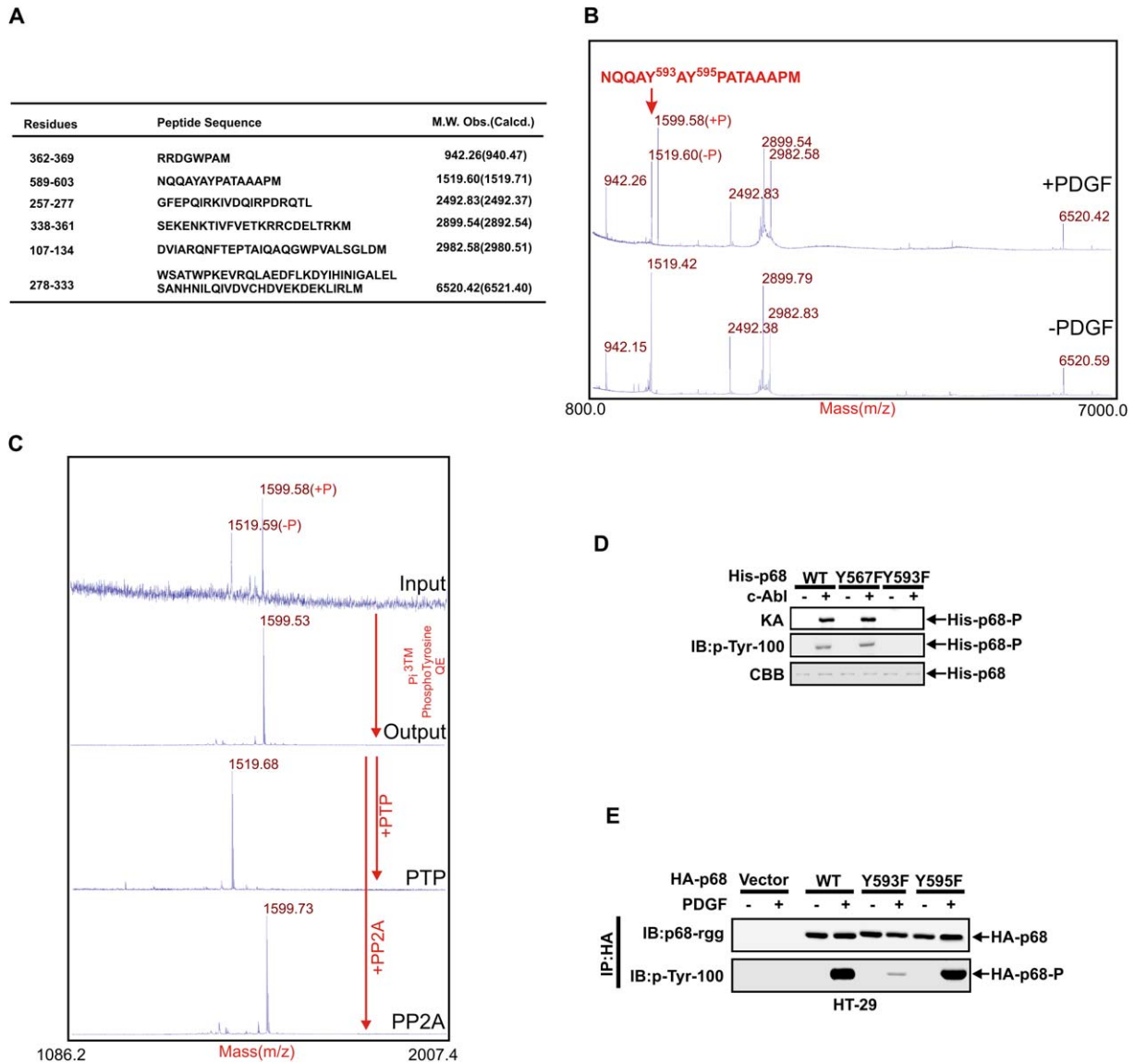
Figure 3. c-Abl Phosphorylates P68 at Y593 in PDGF-Stimulated HT-29 Cells

(A) P68 CNBr cleavage peptides recovered from MALDI spectrum. MW, Obs and Calcd are the observed or calculated molecular weight, respectively.

(B) MALDI mass spectroscopy of CNBr cleaved endogenous p68 with PDGF treatment (upper panel) or without PDGF treatment (bottom panel).

(C) MALDI of CNBr cleaved p68 purified from PDGF-treated cells. The peptide mixture was treated with and purified by Pi^3 phosphotyrosine QE kit (Output), PTP after Pi^3 purification (PTP), PP2A after Pi^3 purification (PP2A). Input shows an untreated peptide mixture.

(D) In vitro phosphorylation of his-p68, WT or Y567F/Y593F mutants, by recombinant c-Abl were detected by the autoradiography (KA), immunoblotting with p-Tyr-100 (IB:p-Tyr-100), and Commassie blue staining (CBB) of the SDS-PAGE of each in vitro kinase reaction.

(E) Tyrosine phosphorylations of exogenously expressed HA-p68s (WT, Y593F, or Y595F mutant) in HT-29 cells with and without (+/−) PDGF treatment were detected by IB using antibody p-Tyr-100 (IB:p-Tyr-100). The HA-p68s were IP from nuclear extracts (IP:HA). The IB:p68-rgg was a loading control.

(Behrens, 2000; Moon et al., 2004). We first carried out in vitro binding analyses with recombinant his-p68s (WT or mutants) and nuclear extracts made from PDGF untreated HT-29 cells. The his-p68s (WT or mutants) were prephosphorylated by c-Abl in vitro. Figure 5A demonstrated that β-catenin coprecipitated with his-p68 WT and the mutant Y567F, but not with the mutant Y593F. The unphosphory-

lated his-p68 did not coprecipitate with β-catenin (Figure 5A). The interaction between p68 and β-catenin was further confirmed by coimmunoprecipitation of exogenously expressed HA-p68 with β-catenin in the nuclear extracts made from PDGF-treated or -untreated HT-29 cells (Figure S3B). Simultaneous examination of the phosphorylation status of the HA-p68 in the coprecipitates revealed
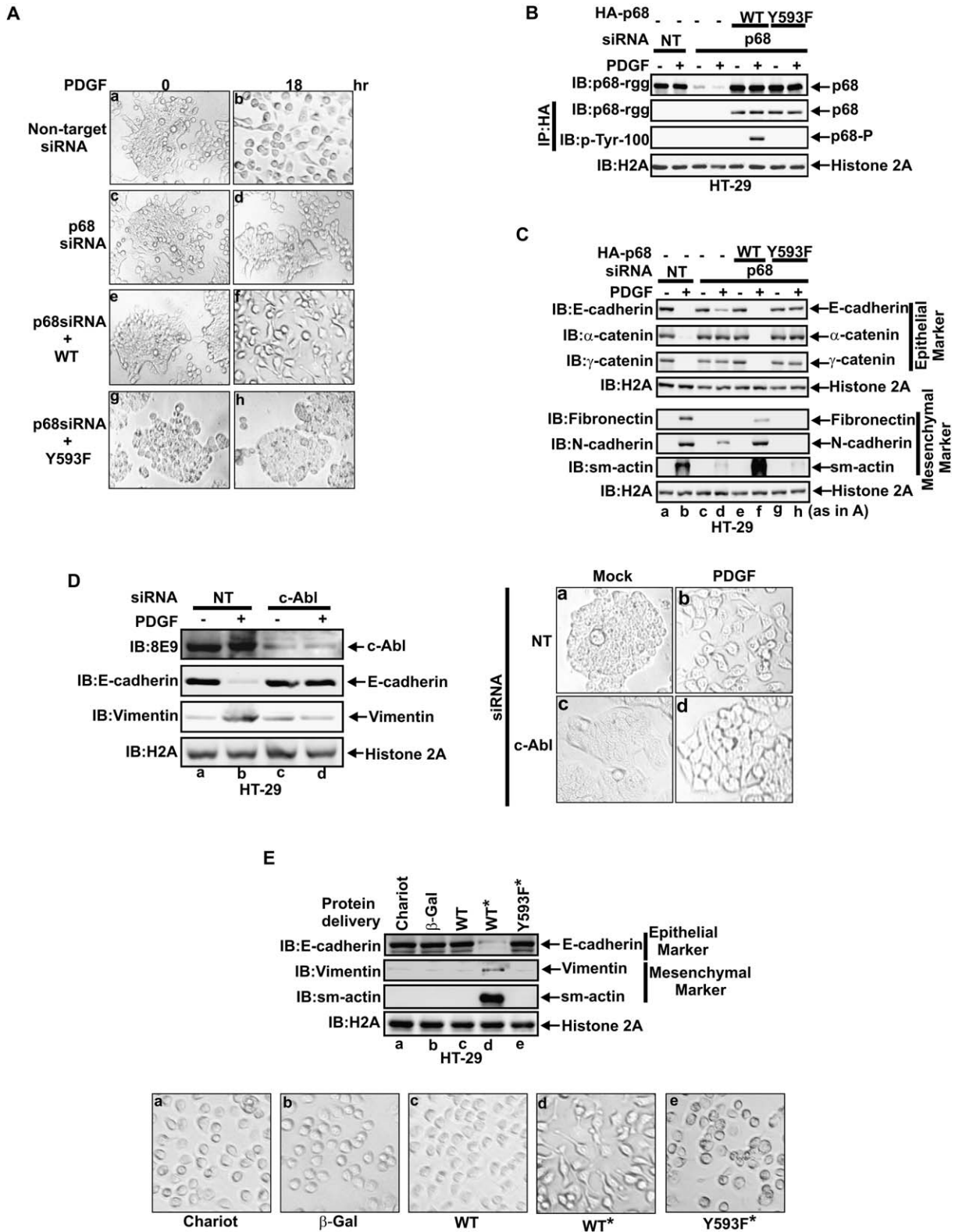
Figure 4. The Y593 Phosphorylation of P68 Is Required for PDGF-Stimulated EMT

(A) The morphologies of HT-29 cells were revealed by phase-contrast microscopy. The cells were treated (18/0 hr) with PDGF-BB. P68 was knocked down (p68/NT) and HA-p68s, WT or Y593F, were expressed.

that p68 was tyrosyl phosphorylated (Figures 5A and S3B). The data suggested that the phosphor-p68 interacted with β-catenin.

P68 interacted with both c-Abl and β-catenin. We next sought to investigate the relationship between these two molecular interactions. The time courses of these two interactions were closely monitored in HT-29 cells that co-expressed FLAG-c-Abl-Myc and HA-p68-his (Figure 5B). First, p68 interacted with c-Abl kinase immediately after PDGF stimulation (within 5 min) and reached a maximum at 30 min, while p68 phosphorylation occurred almost simultaneously with a slight lagging behind. Although the phosphorylation of p68 remained constant over a 180 min time course, the association of p68 with c-Abl started to decline after 60 min of PDGF treatments and was almost completely abolished at 180 min. On the other hand, the coprecipitation of β-catenin with p68 was almost undetectable until 30 min after PDGF treatment. The interaction between β-catenin and p68 did not show any significant decrease over a 180 min time course. The results indicated that p68 was initially phosphorylated by c-Abl and subsequently interacted with β-catenin, consistent with the observation that only phosphor-p68 interacted with β-catenin.

Since the phosphor-p68 interacted with nuclear β-catenin, we proceeded to investigate whether the phosphor-p68 promoted nuclear accumulation of β-catenin. Immunostaining of β-catenin showed that the protein translocated to the cell nucleus in HT-29 cells under PDGF stimulation. This translocation was largely blocked by p68 knockdown. Transient expression of WT p68 restored the pattern of nuclear staining of β-catenin. However, the Y593F-expressing cells demonstrated an exclusive staining of β-catenin in the cell membrane (Figure 5C). The role of p68 Y593 phosphorylation in promoting β-catenin nuclear translocation was further examined by monitoring the β-catenin in nuclear extracts and cell lysate (Figure 5D). In the HT-29 cells without PDGF treatment, there was a very low level of β-catenin in the nucleus. Treatment of cells with PDGF promoted the translocation of β-catenin to the nucleus. The effects of PDGF on enhancing β-catenin nuclear translocation were abolished by p68 knockdown. However, the effects were restored

by exogenous expression of WT p68. The enhancement correlated with the total level of p68 tyrosine phosphorylation. However, the nuclear β-catenin level was slightly decreased after expression of mutant Y593F in the p68 knockdown cells. The observed changes in nuclear β-catenin were not due to a change in β-catenin expression, because the β-catenin level in cell lysate remained almost constant. Thus, our data suggested that phosphorylation of p68 played a role in promoting β-catenin translocation into the cell nucleus. This phenomenon was also observed in HCT-116 cells (data not shown).

β-catenin is a component of cell adherens junctions, connecting E-cadherin to α-catenins and γ-catenins. Repression of E-cadherin and α- γ-catenins may free the membrane β-catenin and increase the cytoplasmic β-catenin. We therefore questioned whether the β-catenin nuclear translocation is a direct or an indirect consequence of disruption of adheren junctions due to repression of E-cadherin. To test this possibility, we examined the effects of the in vitro phosphorylated p68 on β-catenin nuclear translocation in HT-29 cells in which E-cadherin was knocked down (Figure 5E) or E-cadherin was exogenously overexpressed (Figure 5F). Knockdown of E-cadherin alone promoted β-catenin nuclear translocation to some degree. Delivery of the phosphor-p68 dramatically enhanced the β-catenin nuclear translocation in the E-cadherin knockdown cells. On the other hand, β-catenin was translocated to the nucleus in HT-29 cells if the phosphor-p68 was delivered regardless of whether the E-cadherin was overexpressed. Thus, we concluded that β-catenin nuclear translocation was not a direct or an indirect consequence of repression of E-cadherin.

Wnt signaling leads to β-catenin nuclear translocation by blocking the GSK-3β-mediated phosphorylation of β-catenin (Karim et al., 2004). We therefore investigated whether β-catenin nuclear translocation promoted by the phosphor-p68 overlapped with the Wnt signaling pathway. P68 was knocked down in HT-29 cells. The cells were subsequently treated by Wnt-1. It was evident that β-catenin was translocated to the cell nucleus under Wnt-1 treatment regardless of whether p68 was knocked down or not (Figure 6A). The results suggested that β-catenin nuclear translocation promoted by Wnt-1 signaling

(B) Expression and phosphorylation of HA-p68s (WT or Y593F) in HT-29 cells were examined by IP of HA-p68s from nuclear extracts (IP:HA) followed by IBs via antibodies p68-rgg (IB:p68-rgg) and p-Tyr-100 (IB:p-Tyr-100). The cells were treated by nontarget siRNA (NT) or p68 RNAi (p68). The total p68s were monitored by IB via p68-rgg without IP (IB:p68-rgg). The IB:H2A was an internal control.

(C) Expression of epithelial proteins, E-cadherin, α-catenin, and γ-catenin, and mysenchymal proteins, fibronectin, N-cadherin, and sm-actin, were examined by immunoblotting in HT-29 cells with and without (+/−) PDGF treatment and with and without (p68/NT) p68 knockdown. The HA-p68s (WT or Y593F) were expressed. The letters at the bottom indicated the same treatments of cells corresponding to the letter marks in panel (A).

(D) Cellular levels of c-Abl, E-cadherin, and vimetin in the HT-29 cells in which c-Abl was knocked down (c-Abl/NT) were examined by IB using appropriate antibody (indicated). The cells were treated or untreated (+/−) with PDGF-BB. The left panel was examined by IB. IB:H2A was a loading control. The morphologies of HT-29 cells treated correspondingly as described in the left panel were revealed by phase-contrast microscopy (right panel). The letters at the bottom of the left panel indicated the same treatments of cells corresponding to the letter marks on the right panel.

(E) Cellular levels of epithelial marker, E-cadherin, and mysenchymal markers, vimentin and sm-actin, in HT-29 cells were examined by IB using appropriate antibody (indicated). The in vitro phosphorylated his-p68s (WT or Y593F), or unphosphorylated his-p68 WT (WT) were delivered into the cells (upper panel). The morphologies of HT-29 cells treated correspondingly as described in upper panel were revealed by phase-contrast microscopy (lower panel). * The indicated his-tagged recombinant protein was incubated with Abl kinase in vitro and subsequently purified by Ni-TED beads. The letters at the bottom of the upper panel indicated the same treatments of cells corresponding to the letter marks on the bottom panel.
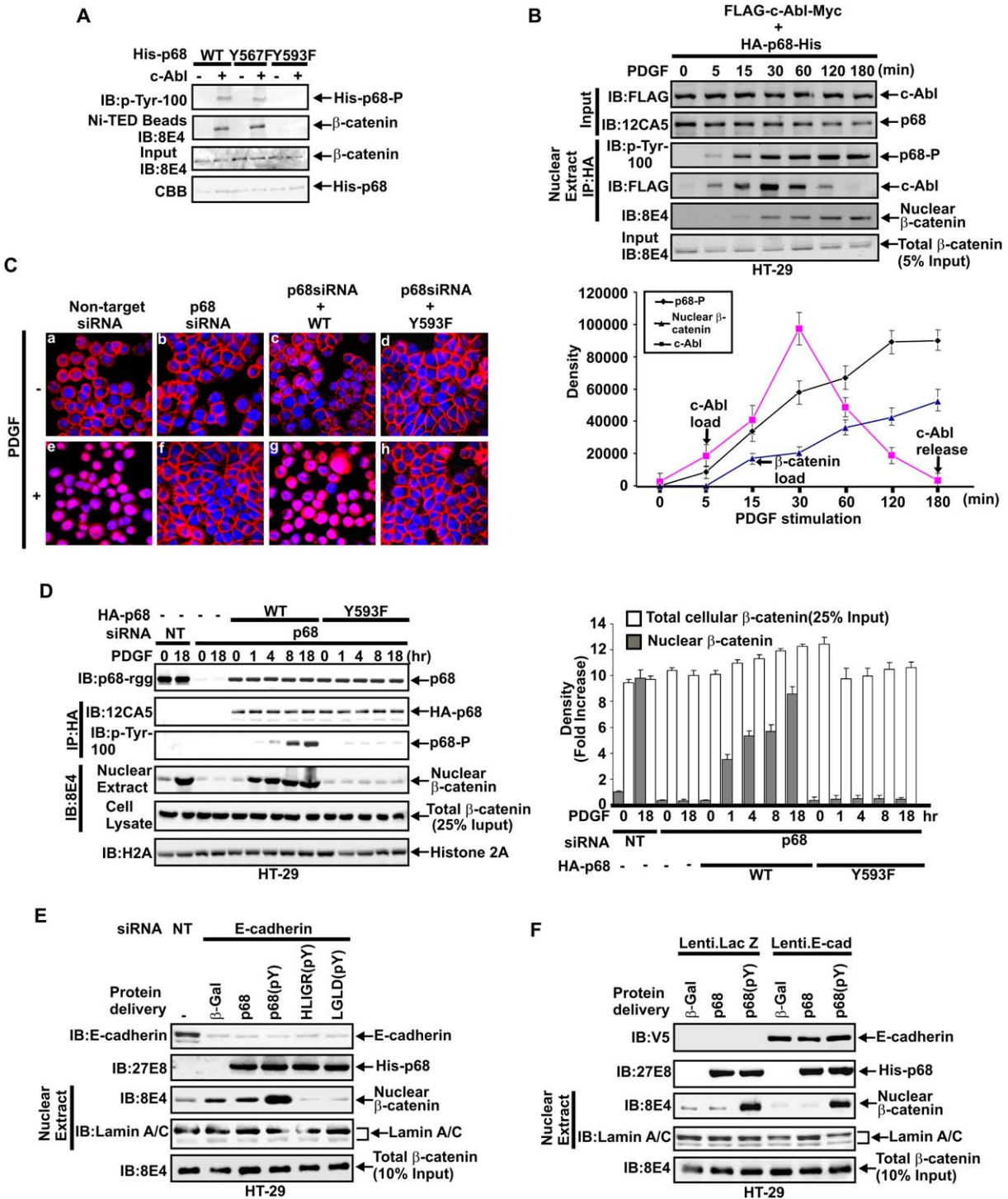
Figure 5. The Y593 Phosphorylated P68 Interacts with β-Catenin in the Nucleus and Promotes β-Catenin Nuclear Translocation

(A) The his-p68s (WT or Y567F/Y593F) were incubated with nuclear extracts made from HT-29 cells. The his-p68s were pretreated or untreated (+/−) by c-Abl for phosphorylation prior to the incubation. The his-p68s were precipitated by Ni-TED beads. The interaction of β-catenin with his-p68 was examined by IB of the precipitates (IB:8E4). The inputs were IB of β-catenin in the extracts without IP. The loading control was Commassie staining of the precipitated his-p68s (CBB).

(B) Time course of effects of PDGF on tyrosine phosphorylation of HA-p68, the HA-p68:FLAG-c-Abl interaction, and the HA-p68:β-catenin interaction in HT-29 cells expressing Flag-c-Abl-Myc and HA-p68-his. The interactions were detected by IP:HA followed by IBs with appropriate antibodies

does not require p68 RNA helicase. Our data also demonstrated that β-catenin nuclear translocation promoted by phosphor-p68 does not require Wnt. Our results, therefore, suggested that β-catenin nuclear translocation promoted by Wnt signaling is phosphor-p68 independent.

To elucidate the mechanism by which the phosphor-p68 promotes β-catenin nuclear translocation, we tested whether the interaction between the phosphor-p68 and β-catenin is required. We employed antibodies against different sequence motifs of β-catenin (Table S1) to block the interaction between the phosphor-p68 and β-catenin and then examine the β-catenin nuclear translocation. The antibodies were delivered to HT-29 cells. The cells were then treated with PDGF. The antibodies against N-terminal (7D11) and region of aa 35–50 (7A7) of β-catenin blocked the interaction. No effects of antibodies against C-terminal or core armadillo repeats on the interaction were observed. Interestingly, an antibody against the N-terminal sequence (aa 27–37) of β-catenin did not block the interaction (Figure 6B). The antibodies that blocked the β-catenin:phosphor-68 interaction also blocked the β-catenin nuclear translocation. The antibodies that did not block the interaction did not block the β-catenin nuclear translocation (Figure 6B). This experiment suggested that the phosphor-p68 may interact with β-catenin at the N terminus (likely aa 35–50 region) and the interaction of the phosphor-p68 with β-catenin is necessary for β-catenin nuclear translocation. Since the phosphorylation sites of β-catenin by GSK-3β are located at Ser33/Ser37/Thr41 (Liu et al., 2002), it is possible that the phosphor-p68 also interacted with the same sites, thereby blocking the GSK-3β phosphorylation. To test this conjecture, we delivered the in vitro phosphor-p68 into HT-29 cells. We then examined the phosphorylation of β-catenin at Ser33/Ser37/Thr41. β-catenin was not phosphorylated in the presence of the phosphor-p68 (Figure 6C). Lack of the β-catenin phosphorylation was not due to downregulation or inactivation of GSK-3β as revealed by the fact that there were no significant changes in the cellular level and activity of GSK-3β (Figure 6C). The inhibition of GSK-3β-mediated β-catenin phosphorylation by the phosphor-p68 was further confirmed by in vitro kinase assays with recombinant his-p68, GSK-3β, and β-catenin (Figure 6D).

The above results suggested a direct role of the phosphor-p68 in promoting β-catenin nuclear translocation. P68 must localize to the cytoplasm to fulfill this role. To test whether the phosphor-p68 localized to cytoplasm, we made cytoplasmic extracts from the PDGF-treated and -untreated HT-29 cells. Immunoblotting detected p68 in the extracts made from PDGF-treated cells but not in the extracts made from PDGF-untreated cells (Figure 6E). The results suggest that p68 is localized to the cytoplasm.

The Phosphor-P68 "Displaces" Axin from β-Catenin Facilitating β-Catenin Nuclear Translocation

P68 RNA helicase is an ATP-dependent RNA helicase (Huang and Liu, 2002). To test whether the ATPase/helicase activities of p68 are required for the function of the protein in mediating the EMT, we generated two p68 mutants RGLD (helicase core motif V) → LGLD and HRIGR (helicase core motif VI) → HLIGR (referred to as LGLD and HLIGR, respectively). The mutations abolished ATPase and RNA-unwinding activities (Lin et al., 2005). β-catenin nuclear translocation was analyzed in the cells in which the in vitro phosphorylated his-p68s (WT or mutants) were delivered. Mutations that abolished ATPase/helicase activities of p68 also inhibited β-catenin nuclear translocation (Figure 5E).

Requirement of ATPase activity for β-catenin nuclear translocation is intriguing. To understand why the ATPase activity was required, we probed proteins that associated with cytoplasmic β-catenin in HT-29 cells with and without PDGF treatment. Two proteins (at ∼120 and ∼50 kDa) associated with β-catenin in PDGF-untreated cells but not in the PDGF-treated cells. A protein (at ∼70 kDa) associated with β-catenin in PDGF-treated cells but not in the PDGF-untreated cells (Figure 6F). The three proteins were identified by MOLDI-TOF/TOF (Figure S4). The ∼120 kDa band was Axin, the ∼70 kDa band was p68 RNA helicase, and the ∼50 kDa band was GSK-3β. We further verified the identity of the proteins by immunoblotting using antibodies against Axin, p68, and GSK-3β (Figure 7A). Similar experiments were also carried out with cytoplasmic extracts made from p68 knockdown HT-29 cells. Unlike in the cells in which p68 was not knocked down, the Axin and GSK-3β associated with the cytoplasmic β-catenin (Figure 7B). These results indicated that Axin and GSK-3β only associated with cytoplasmic β-catenin in the absence of the phosphor-p68. The results suggest that, upon interaction with β-catenin, the phosphor-p68 displaced Axin-GSK-3β from the cytoplasmic β-catenin

(indicated). The top input panels were the IB with indicated antibodies without IP. The input of β-catenin was IB of β-catenin in 5% extracts without IP. The bottom panel is the quantitation of average four times of the IB signals. Error bars represent standard deviation (n = 4).
(C) Immunofluorescence staining of β-catenin in HT-29 cells, in which p68 RNA helicase was knocked down and p68s (WT or Y593F mutant) were overexpressed in p68 knockdown cells. The cells were treated/untreated (+/−) with PDGF-BB. The red signal represents staining of β-catenin.
(D) Immunoblotting of the β-catenin level in the nuclear or whole cell lysates of HT-29 cells after various PDGF treatment times (0–18 hr). P68 was knocked down and HA-p68s (WT or Y593F) were expressed (as indicated). The left panel is the IBs with appropriate antibodies (indicated). The right panel is the quantitation of the average of four IB signals. Error bars represent standard deviation (n = 4).
(E and F) Immunoblot analyses of E-cadherin (IB:E-cadherin) and his-p68 (IB:27E8) in whole cell extracts and β-catenin in nuclear extracts (IB:8E4). The in vitro phosphorylated/unphosphorylated his-p68s (WT [p68(pY)/p68], or [HLIGR(pY), LGLD(pY)] mutants) or control β-Gal (indicated) was delivered to the cells. The cells were treated with non-target RNAi (NT) or RNAi target E-cadherin (E-cadherin) in panel (E), or E-cadherin (Lenti.E-cad) or LacZ (Lenti.LacZ) was expressed in the cells using the lentiviral system in panel (F). IB:Lamin A/C and 10% of total cellular β-catenin (IB:8E4) were controls.
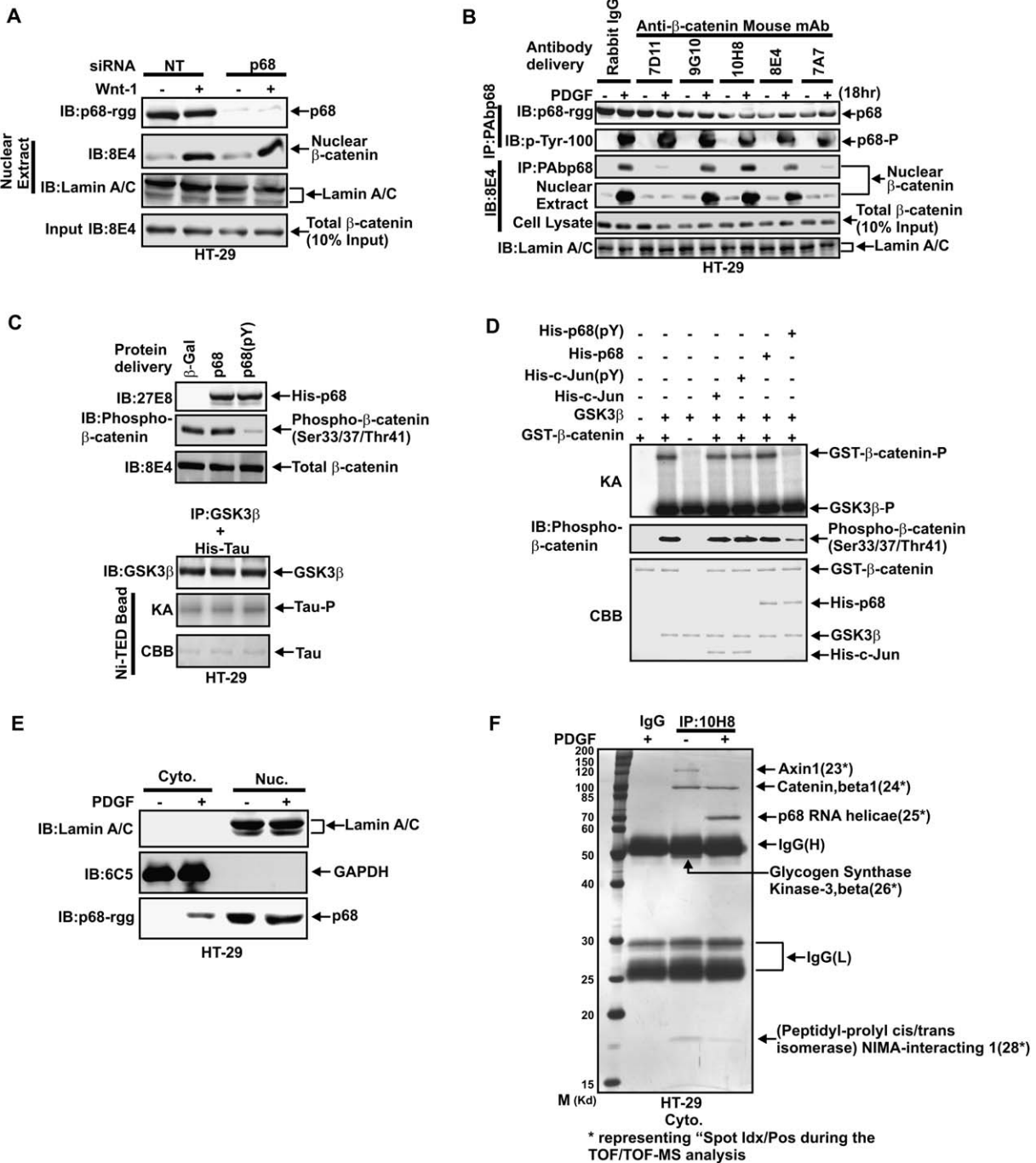
Figure 6. The Y593 Phosphorylated P68 Promotes β-Catenin Nuclear Translocation via Wnt-Independent Pathway

(A) Nuclear β-catenin was examined by IB of nuclear extracts made from p68 knockdown (p68/NT) HT-29 cells with and without (+/−) Wnt-1 stimulation (10 ng/ml in culture medium). The cellular level of p68 was shown by IB using antibody p68-rgg (IB:p68-rgg). The IB:LaminA/C was a loading control. The input was IB of β-catenin in 10% of whole cell extracts.

(B) Nuclear β-catenin was examined by IB of nuclear extracts of HT-29 cells (panel 4 from top). The tyrosine phosphorylation of p68 (panel 2 from top) and β-catenin:p68 interaction (panel 3) were monitored by IB of coIPs (IP:PAbp68) using appropriate antibodies (indicated). Monoclonal antibodies (indicated) against β-catenin were delivered to HT-29 cells. The cells were treated or untreated (+/−) with PDGF. Rabbit IgG was used as a negative control. IB:LaminA/C was a loading control. The input was IB of β-catenin in 10% of whole cell extracts.

(C) Analyses of the his-p68, phosphor-β-catenin, and total β-catenin in whole cell extracts made from HT-29 cells by IB using indicated antibody. The phosphorylated/unphosphorylated [p68(pY)/p68] his-p68 was delivered to the cells (upper panel). The lower panel is kinase assay by incubating re-combinant his-tagged Tau with the IP GSK3β from the HT-29 cells treated as upper panel. The his-Tau was pulled down by Ni-TED beads and

complex, therefore facilitating β-catenin nuclear translocation. In this model, the ATPase activity of p68 may be required for the Axin-GSK-3β displacement. We therefore tested whether the Axin-GSK-3β dissociated from the cytoplasmic β-catenin by the in vitro phosphorylated recombinant his-p68. β-catenin was immunoprecipitated from cytoplasmic extracts made from PDGF-untreated HT-29 cells. Immunoblotting demonstrated that p68 was not present in the precipitates. The in vitro phosphorylated p68 was then added to the precipitates in the presence and absence of ATP. It was evident that Axin was dissociated from the precipitates only when the in vitro phosphorylated p68 was added in the presence of ATP (Figure 7C). If the in vitro phosphorylated HLIGR was added to the precipitates, Axin was not dissociated even in the presence of ATP (Figure 7C). However, when the phosphor-p68 or phosphorylated HLIGR was added in the absence of ATP, GSK-3β was coprecipitated with the β-catenin (Figure 7C). Thus, our data clearly suggested the following: (1) the phosphor-p68 binds β-catenin and blocks its phosphorylation by GSK-3β, and (2) the phosphor-p68 "displaced" Axin from cytoplasmic β-catenin. To further confirm the role of the phosphor-p68 in "displacing" Axin, the p68s (WT, Y593F, LGLD, and HLIGR) were overexpressed in HT-29 cells using the lentiviral system. Coimmunoprecipitation demonstrated that the exogenously expressed WT, HLIGR, and LGLD interacted with cytoplasmic β-catenin in PDGF-treated cells. However, the Y593F mutant did not (Figure 7D). In the Y593F-expressing cells, both GSK-3β and Axin coimmunoprecipitated with the cytoplasmic β-catenin. However, in the HLIGR- or LGLD-expressing cells, Axin coimmunoprecipitated with β-catenin and GSK-3β did not (Figure 7E). To determine whether displacing Axin facilitates β-catenin nuclear translocation, we examined nuclear β-catenin in HT-29 cells, where Axin was knocked down by RNAi. It was evident that knockdown of Axin led to accumulation of β-catenin in the cell nucleus (Figure 7F).

The β-Catenin Nuclear Translocation Is Required for EMT
We now asked whether the β-catenin nuclear translocation played a role in EMT. We first delivered the phosphor-p68 and the antibodies against β-catenin, 8E4, and 7A7 into HT-29 cells. In the presence of the phosphor-p68 and the antibody 8E4, the cells underwent EMT as demonstrated by changes in morphology (Figure 8A) and EMT markers (Figure 8B). However, the EMT was blocked by the antibody 7A7 (Figures 8A and 8B), and β-catenin was not translocated to the cell nucleus in the presence of the antibody 7A7. The nuclear β-catenin activates a number of genes by complex with LEF/TCF (Eastman and Grosschedl, 1999). To test whether this pathway is required for the EMT, the TCF-1 was knocked down. The TCF-1 knockdown (over 70%) was effective as demonstrated by blocking the Wnt-1-stimulated β-catenin nuclear translocation (Figure 8C). We then examined the effects of TCF-1 knockdown on EMT in the presence of the in vitro phosphor-p68. First, the β-catenin nuclear translocation was not affected by TCF knockdown (Figure 8E). However, EMT was almost completely blocked as revealed by the changes in morphology (Figure 8E) and EMT markers (Figure 8D). Thus, we concluded that the β-catenin nuclear translocation and subsequent complex with LEF/TCF was required for EMT mediated by the phosphor-p68.

DISCUSSION

Nuclear translocation of β-catenin is a central player in the Wnt signaling pathway, which can trigger cell proliferation, differentiation, and migration (Nelson and Nusse, 2004). The phosphor-p68 promotes cytoplasmic β-catenin nuclear translocation via a Wnt-independent pathway. This is a newly discovered pathway for β-catenin nuclear translocation. We propose a model to illustrate the role of the phosphor-p68 in promoting EMT through facilitating β-catenin nuclear translocation (Figure 8F). Binding of the phosphor-p68 to β-catenin precludes the contacts of GSK-3β with β-catenin, thus preventing β-catenin from phosphorylation. However, blocking the contacts of β-catenin with GSK-3β is not sufficient to promote β-catenin nuclear translocation, as ATPase/helicase activity of the phosphor-p68 was not required for dissociating GSK-3β, but the activity is required for β-catenin nuclear translocation. Our experiments showed that the ATPase/helicase activity of the phosphor-p68 is employed to "displace" Axin from cytoplasmic β-catenin. Axin is an important protein factor that negatively regulates β-catenin nuclear translocation. It was shown that Axin functioned to anchor β-catenin in the cytoplasm (Tolwinski and Wieschaus, 2004). Thus, it is clear that the phosphor-p68 facilitates β-catenin nuclear translocation by both blocking GSK-3β phosphorylation and displacing the negative regulator Axin.

subjected to autoradiography (KA) or Coommasie blue staining (CBB). The top on the lower panel was the expression of GSK-3β examined by IB from the whole cell extracts.
(D) In vitro phosphorylations of β-catenin by recombinant GSK-3β in the presence of phosphorylated/unphosphorylated his-p68s or his-c-Jun were determined by the autoradiography (KA), immunoblotting with phosphor-β-catenin antibody (IB), and Coommasie blue staining (CBB) of the SDS-PAGE of each in vitro kinase reaction.
(E) IB analyses of p68 in cytoplasmic (cyto.) or nuclear (Nuc.) extracts made from PDGF treated/untreated (+/−) HT-29 cells. IB:Lamin A/C and IB:6C5 are the controls for nuclear or cytoplasmic proteins respectively.
(F) Silver staining the SDS-PAGE of coIP of cytoplasmic β-catenin. The β-catenin was precipitated from cytoplasmic extracts made from PGDF-treated or -untreated (+/−) HT-29 cells. Mouse IgG was used as a control pull down. The proteins that coprecipitated with β-catenin (identified by MALDI-TOF/TOF) were indicated on the right side.
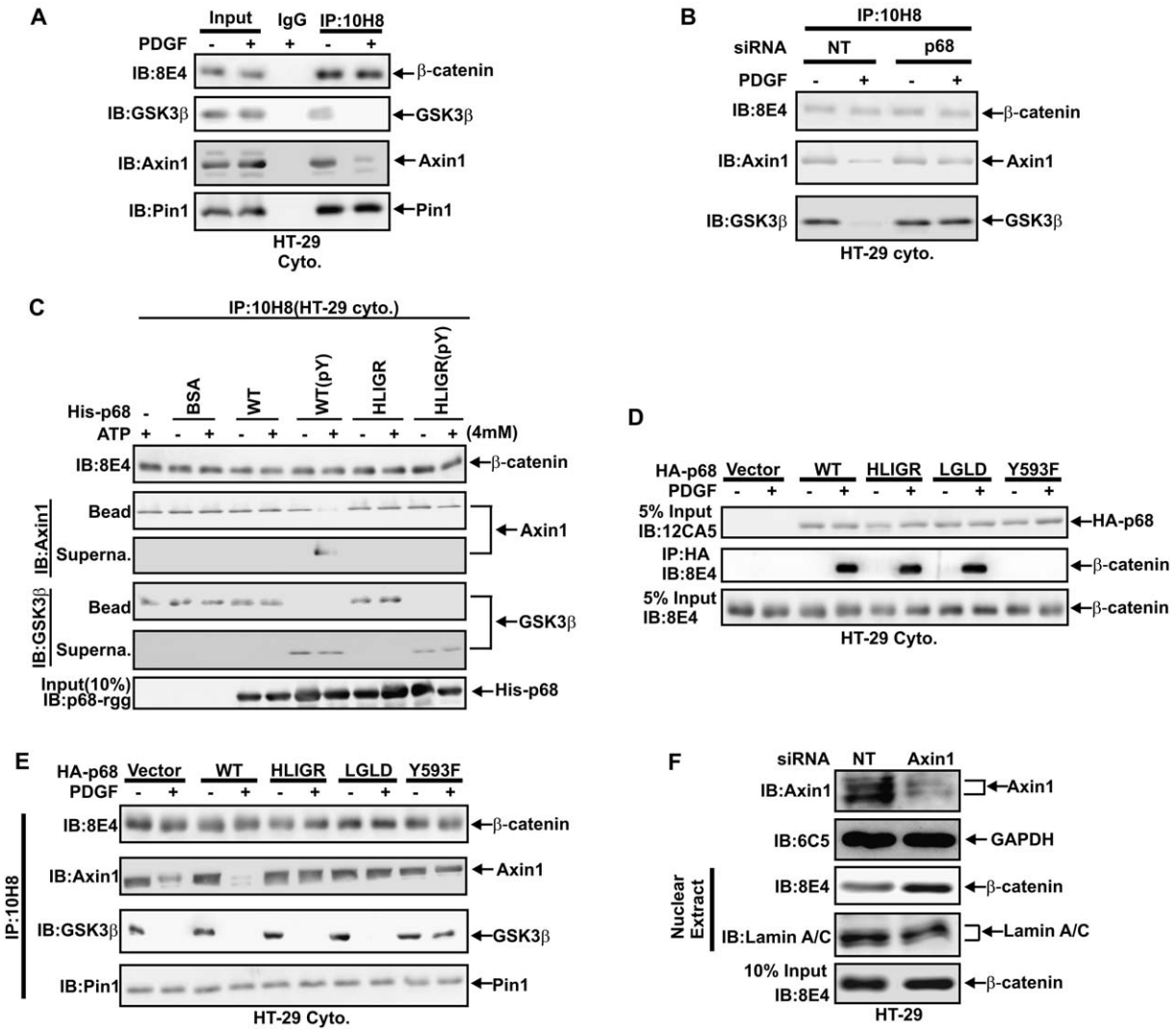
Cell

Figure 7. Phosphor-P68 Displaces Axin1 from Cytoplasmic β-Catenin

(A and B) CoIP of GSK-3β, Axin1, and Pin1 with β-catenin in cytoplasmic extracts made from PDGF-treated or -untreated (+/−) HT-29 cells were detected by IBs using appropriate antibodies (indicated). The cytoplasmic β-catenin was IPed by antibody 10H8. In panel (A), IPs by mouse IgG (IgG) was control IP. Inputs were the IBs without IP. In panel (B), the cells were treated by p68 siRNAs (p68) or nontarget siRNA (NT).

(C) Associations (beads) and dissociation (Superna.) of Axin1 and GSK-3β with the β-catenin that was immunoprecipitated from cytoplasmic extracts made from PDGF-untreated HT-29 cells were detected by IBs using antibodies against Axin1 or GSK-3β (indicated). The coIP complexes were incubated with the in vitro phosphorylated (pY) or unphosphorylated his-p68s (WT or HLIGR mutant) and in the presence or absence of ATP (+/−). IB:8E4 and IB:p68-rgg were loading controls.

(D) CoIPs of cytoplasmic β-catenin with HA-p68s (WT or mutants) were detected by IBs using antibody 8E4 (IB:8E4). The HA-p68s was IPed from cytoplasmic extracts of PDGF-treated or -untreated (+/−) HT-29 cells using anti-HA antibody (IP:HA). Inputs were the IBs of 5% extracts without IP.

(E) CoIPs of Axin1, GSK-3β, and Pin1 with cytoplasmic β-catenin were detected by IBs using appropriate antibodies (indicated). The β-catenin was IPed from cytoplasmic extracts of PDGF-treated or -untreated (+/−) HT-29 cells using antibody 10H8 (IP:10H8). HA-p68s (WT or mutants) was exogenously expressed in the cells (indicated). IB:8E4 was a control.

(F) Nuclear β-catenin (IB:8E4) was examined by IB of nuclear extracts made from HT-29 cells, in which Axin1 was siRNA knocked down (NT/Axin) (IB:Axin). The IB:6C5 and IB:Lamin A/C and input β-catenin were loading controls.

Since Axin is a scaffold protein that holds β-catenin and GSK-3β in a β-catenin degradation complex, it is somewhat surprising that the p68 mutants that lack ATPase activity dissociate GSK-3β without dissociation of Axin. The best explanation is that phosphor-p68 and mutants physically contact Axin (the displacing target of p68). The contact site is very close to the Axin:GSK-3β binding site. It is conceivable that, although the phosphor-p68 mutants (HLIGR and LGLD) cannot displace Axin, the mutants physically contact Axin. This contact competes with the

Axin:GSK-3β interaction. Similar competition is observed between the synaptic scaffolding molecule (S-SCAM) and GSK-3β. Both molecules compete to bind to Axin in neuro cells (Hirabayashi et al., 2004).

To promote cytoplasmic β-catenin nuclear transloca-tion, the phosphor-p68 must be localized to the cyto-plasm. The molecular mechanism underlining the translo-cation of p68 is an open question in our proposed model (Figure 8F). Our current hypothesis is that the tyrosine phosphorylation may help p68 translocate to the cyto-plasm. P68 was first observed predominantly localized in the cell nucleus (Nicol et al., 2000). Recently, the protein was shown to transiently localize to the cytoplasm (Ros-sow and Janknecht, 2003). Our experiments also de-tected the cytoplasmic p68. Interestingly, our data clearly demonstrated that p68 translocated to the cytoplasm of HT-29 cells upon PDGF stimulation (Figure 6E). Whether this result suggests that only the phosphor-p68 localizes to the cytoplasm requires further tests.

The requirement of ATPase/helicase activities of p68 in β-catenin nuclear translocation brought about an interest-ing question. What is the substrate(s) that stimulates ATPase activity of the phosphor-p68? Our experiments suggest the "displacing" role of the phosphor-p68 in dis-sociating Axin from cytoplasmic β-catenin. This observa-tion strongly suggests a novel protein-dependent ATPase activity of p68 RNA helicase. Although, it was suggested that the DExH/D box RNA helicases may function as RNA unwindase to dissociate RNA-protein interactions (Jankowsky et al., 2001). This is the first example of a DExD/H box RNA helicase with the activity to dissociate protein-protein interactions. An interesting question is whether the Y593 phosphorylation of p68 is required for the protein-dependent "displacing" activity. It is possible that p68 RNA helicase may gain a "protein-dependent ATPase" activity after the tyrosine phosphorylation. It is also possible that the "protein-dependent ATPase" does not depend upon phosphorylation. The activity is sub-strate specific. Phosphorylation of p68 at Y593 facilitates the recognition of the substrate β-catenin. In this regard, it will be interesting to identify potential target protein(s) that will stimulate the protein-dependent ATPase activity of unphosphorylated p68.

The phosphorylation of p68 by c-Abl kinase is intriguing. It was demonstrated that the plasma membrane and cyto-plasm pools of c-Abl kinase were activated by PDGF. It was shown that Src kinase is activated upon the PDGF treatment in fibroblast. The activated src will subsequently phosphorylate and activate c-Abl of plasma membrane and cytoplasm pools (Lu et al., 2001; Plattner et al., 1999). Consistently, we previously observed that Src was required for p68 tyrosine phosphorylation (Yang et al., 2005). The question is, "Where is p68 phosphory-lated by c-Abl?" Since p68 interacted with c-Abl in the cell nucleus, and the recombinant p68 was phosphory-lated by immunoprecipitated c-Abl from nuclear extracts, our current hypothesis is that p68 is phosphorylated by c-Abl in the nucleus. Our data indeed showed that nuclear

c-Abl was activated in two epithelial cell lines upon PDGF stimulation (Figure 2B and data not shown). On the con-trary, Plattner and coworkers reported that the nuclear c-Abl is not activated by PDGF in NIH-3T3 cells (Plattner et al., 1999). One explanation for the contradictory obser-vations is that the activated cytoplasmic pool of c-Abl may migrate to the cell nucleus to phosphorylate p68. It is also possible that the activation of nuclear c-Abl kinase is cell- and tissue-type specific. Although c-Abl has been implicated in a number of cellular processes, including dif-ferentiation, stress responses, cell division, and cell adhe-sions, our experiments suggest that c-Abl may also play a role in mediating the effects of PDGF to promote EMT. Another interesting phenomenon is that the Y593F mutant does not seem to be a simple loss-of-function mutant. In a number of overexpression experiments, the mutant competed with the endogenous protein. We believe that the mutant competes with the WT p68 for c-Abl binding (Figure S1C). The consequence of binding to c-Abl by the Y593F mutant is inhibition of the c-Abl activity, which in turn blocks the phosphorylation of endogenous p68.

P68 RNA helicase is implicated in tissue and organ de-velopmental processes, including early organ maturation (Stevenson et al., 1998), embryogenesis (Seufert et al., 2000), and wound healing (Kahlina et al., 2004). The find-ings that phosphorylation(s) of p68 promoted the EMT certainly provide a cellular and molecular basis for the de-velopmental role(s) of the protein. More interestingly, we observed a substantially higher level of tyrosine phosphor-ylation(s) of p68 in metastatic cancer tissue samples than those in primary sites. No tyrosine phosphorylation of p68 occurred in normal tissue samples (unpublished observa-tions). Although we do not know whether the higher level of tyrosine phosphorylation in metastatic cancer tissues was due to phosphorylation at Y593, given that EMT is vi-tal for an epithelial tumor to lose its polarity and progress toward tumor invasion and metastasis (Kang and Mas-sague, 2004; Yang et al., 2004a), and that the p68 Y593 phosphorylation promoted cell invasion, it is tempting to speculate that the tyrosine phosphorylation at Y593 may promote cancer metastasis.

EXPERIMENTAL PROCEDURES

Reagents and Antibodies
All recombinant growth factors, c-Abl kinase, GST-β-catenin, and his-GSK3β, his-Jun, and Tau proteins were obtained from Upstate, Active motif, PeproTech, and Biomol, respectively. The c-Abl inhibitor STI-571 is a gift from Novartis. The antibodies used in this study are listed in Table S1.

DNA Constructs, Recombinant Protein Expression, and Purification
The HA-tagged p68 expression plasmid was constructed by subclon-ing ORF of p68 from p68 expression vector (Huang and Liu, 2002) into pHM6 vector (Roche). To construct Myc-tagged c-Abl expression vec-tor, ORF of c-Abl was amplified from an mRNA pool of human HeLa S3 cells using primer, forward 5′-AAGGAAAAAAGCGGCCGCGCAGCAGC CTGGAAAAGTACT-3′, reverse 5′-CGGGATCCCCTCTGCACTATGTCA CTGA-3′. The cDNA of ORF was subcloned into pFLAG-myc-CMV -20
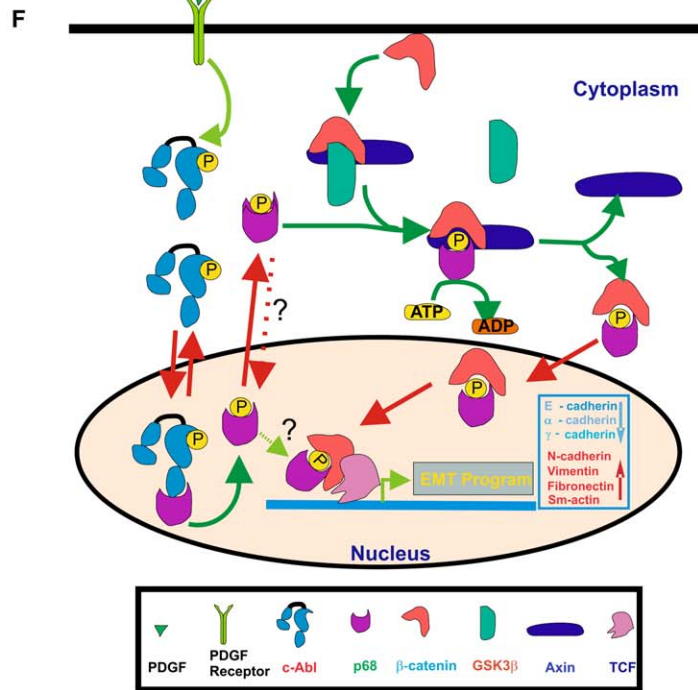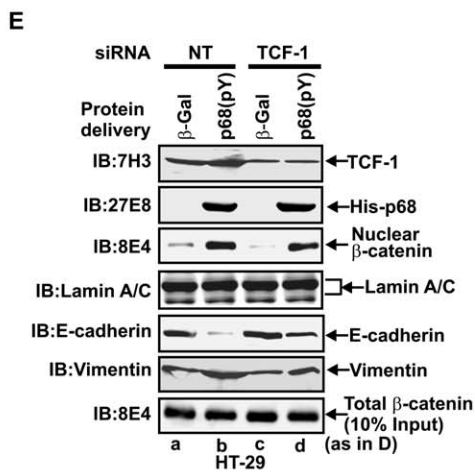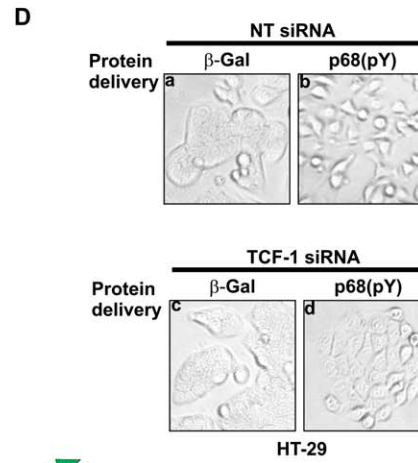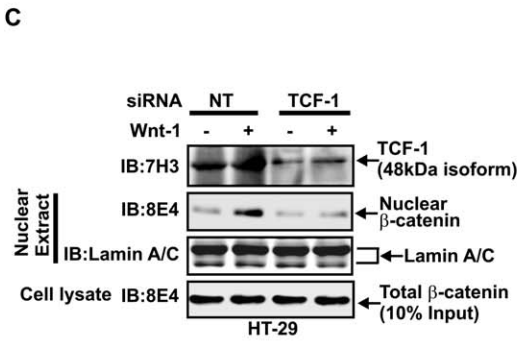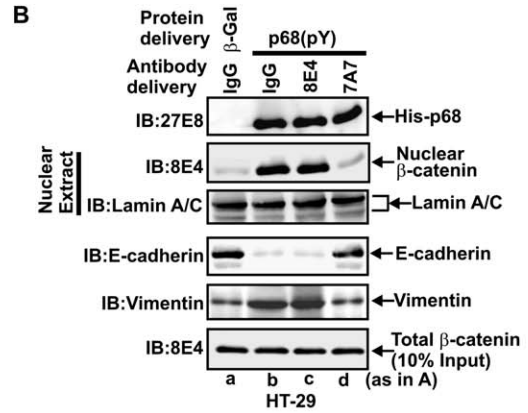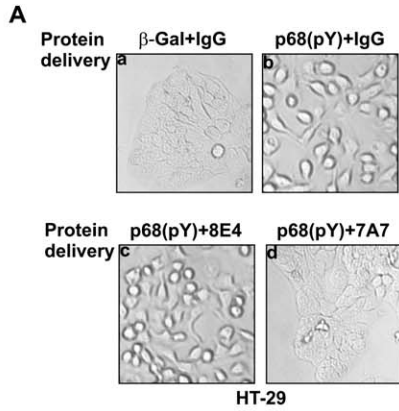
vector at Not I/BamH I sites (Sigma). Site-directed mutagenesis was performed with mutation kits QuickChange site-directed mutagenesis (Stratagene). The mouse E-cadherin cDNA was provided by Dr. Gerhard Christofori at the Research Institute of Molecular Pathology, Vienna, Austria and subcloned into the pLenti6/TOPO vector. All cloned DNA and mutations were confirmed by DNA sequencing. Recombinant 6Xhis-p68 and its derived mutants and other recombinant proteins were expressed in bacterial BL21 and affinity purified on Nickel-TED beads by the same procedure described in our previous report (Yang and Liu, 2004; Yang et al., 2004b).

Cell Culture and RNA Interference

A549, HeLa, HepG2, HT-29, HCT116, K562, SW620, T98G, WM115, and MDCK cells were purchased from ATCC and grown by following the vendor's instructions. All DNAs or RNAs transfections were performed using Lipofectamine 2000 by following the manufacturer's instructions (Invitrogen). For the siRNA experiments, cells were grown to 50% confluence and transfected with siRNA (200 pmole). The duplex RNA oligonucleotides for RNAi were purchased from Dharmacon. siRNA olignucleotides against p68 (sense: GCAAGUAGCUGCUG AAUAUUU; antisense: 5'-PAUAUUCAGCAGCUACUUGCUU) were purchased from Dharmacon Individual siGENOME™ Duplex. For transient expression of WT p68 or mutants in p68 knockdown cells, the cells were transfected with the indicated plasmid DNA 24 hr after the cells were transfected with siRNA and harvested after an additional 48 hr of incubation. For treatment of cells with PDGF-BB, the cells were serum starved for 24 hr before the agents (20 ng/ml or doses indicated in figures) were added to the cell-culture medium. After the treatment of 30 min or the time indicated, the cells were immediately harvested for nuclear extracts preparations.

Subcellular Extracts Preparation, Immunoprecipitation, and Immunoblot Analyses

All nuclear extracts, cytoplasm, or cell lysate were made freshly after appropriate treatments (indicated in figures) by using cell extracts kits (Activemotif). The protein concentration of the extracts was determined using the Bradford assay (Bio-Rad). Immunoprecipitation experiments and immunoblot analyses were performed as described in previous studies (Liu et al., 1998). The blotting signals were detected using SuperSignal West Dura Extended Duration Substrate (Pierce). Quantitative analyses of immunoblot signals were obtained via densitometry analyses using NIH Scion Image Software.

In Vitro Kinase Assay and Immune Complex Kinase Assay

The in vitro kinase assays were performed as described in previous studies (Yang and Liu, 2004; Yang et al., 2004b).

For the immune complex kinase assay, endogenous c-Abl or exogenous FLAG-c-Abl proteins were immunoprecipitated from nuclear extracts made from cells with or without PDGF treatments. The immunoprecipitates were washed twice in RIPA buffer, once in high-salt buffer (100 mM Tris-HCl [pH 7.5], 500 mM NaCl) and once in kinase buffer. Kinase reactions were performed for 30 min at 30°C after adding 1 μg appropriate substrates and 100 μM cold ATP and 5 μCi

$[^{32}P]$-γ-ATP. Samples were separated by SDS-PAGE and analyzed by autoradiography or immunoblotting with phosphotyrosine-specific antibody.

Immunofluoresence and Imaging

The cells were seeded on chambered microslides (BD Biosciences) and treated as indicated. The cells were washed, fixed, and permeabilized with 4% formaldehyde and 0.1% Triton X-100 in 1× PBS. The cells were then blocked with Image-iT FX signal enhancer (Molecular Probes), and subsequently incubated with appropriate antibodies for 1 hr. After extensive wash, the samples were incubated with Alexa Fluor 488 or 555 goat anti-mouse IgG (Molecular Probes) (1:1000) to stain primary antibodies. Microslides were washed and mounted in ProLong® Gold antifade reagent with DAPI (Molecular Probes) and viewed using a Zeiss LSM510 Confocal Microscope.

MALDI-Mass

The p68 was immunopurified from PDGF-treated or -untreated HT-29 cells and electrotransferred to nitrocellulose membranes. The p68 band was excised out from the membrane. The membrane was then incubated with 100 mg/ml CNBr (Sigma) in 70% formic acid for 48 hr at room temperature. After washes with 2×500 μl of water and drying, the resultant peptides mixture was then dissolved in 70 μl water and analyzed by MALDI. To enrich phosphotyrosine-containing peptides, the CNBr digestion mixtures were further purified by Pi^3 PhosphoTyrosine-QE Kit (Nest Group Inc.). The purified peptide mixtures were further treated with PTP or PP2A. The mixtures were analyzed by mass spectroscopy.

Protein Delivery

The recombinant proteins or antibodies (indicated) were delivered into HT-29 cells using the Chariot protein delivery kit (Active motif). Briefly, 1 μg of protein (in 100 μl PBS) was mixed with 6 μl of Chariot (in 100 μl H$_2$O). The mixture was incubated at room temperature to allow complex to form. The cells were washed with serum-free medium. The Chariot/protein complex was added to the cells with an additional 400 μl of serum-free medium. The cells were then incubated at 37°C for 2 hr to allow protein internalization.

Expression of P68s and E-Cadherin by Lentiviral System

Stable overexpression of HA-tagged p68 WT or Y593F, HLIGR, and LGLD mutants were carried out using the ViralPower lentiviral expression system (Invitrogen) by following the manufacturer's instructions. The ORFs of p68 wild-type, Y593F, HLIGR, and LGLD mutants with N-terminal HA-tag were cloned into pLenti6/TOPO (Invitrogen). The mouse E-cadherin ORF was subcloned into the pLenti6/TOPO vector fused with C-terminal V5 tag. The infections of HT-29 cells with the lentiviruses that carry p68s or E-cadherin were carried out in the presence of 6 μg/mL of polybrene and 10 mM HEPES. Following transduction, the HT-29 cells were selected by 8 μg/mL of Blasticidin (Invitrogen).

Figure 8. β-Catenin Nuclear Translocation and TCF Were Required for EMT

(A and B) The morphologies of HT-29 cells (A) and IB (B) of cellular his-p68, E-cadherin, vimentin, and nuclear β-catenin using appropriate antibodies (indicated). The antibodies (indicated) and in vitro phosphor-p68 were codelivered to the cells. Delivery of IgG + β-Gal was a negative control. IB:LaminA/C was a loading control. The input was IB of β-catenin in 10% of whole cell extracts. The letters at bottom (B) indicated the same treatments of cells corresponding to the letter marks in panel (A).

(C) IB of cellular TCF-1 and nuclear β-catenin in TCF-1 knockdown (TCF-1/NT) HT-29 cells with/without (+/−) Wnt-1 stimulation. IB:LaminA/C was a loading control. The input was IB of β-catenin in 10% of whole cell extracts.

(D and E) The morphologies of HT-29 cells (D) and IB (E) of cellular his-p68, TCF-1, E-cadherin, vimentin, and nuclear β-catenin using appropriate antibodies (indicated). The cells were treated TCF-1 nontarget RNAi (NT) or RNAi target TCF-1 (TCF-1). The in vitro phosphor-p68 were delivered to the cells. Delivery of β-Gal was a negative control. IB:LaminA/C was a loading control. The input was IB of β-catenin in 10% of whole cell extracts. The letters at bottom (E) indicated the same treatments of cells corresponding to the letter marks in (D).

(F) A hypothetical model schematically illustration of the function of the phosphor-p68 in promoting EMT in response to PDGF stimulation.

In Vitro Protein Complex Dissociation Assays

β-catenin was immunoprecipitated from cytoplasmic extracts made from HT-29 cells using antibody 10H8. Immunoprecipitates were extensively washed with RIPA buffer (supplemented with protease inhibitor and phosphatase inhibitor cocktail) 8 times and subsequently incubated with 250 ng indicated recombinant proteins in 50 μl ATPase assay buffer containing 20 mM Tris-HCl, pH 7.5, 200 mM NaCl, 1 mM $MgCl_2$, and 5 mM DTT with and without 4 mM ATP. The dissociation reactions were incubated at 37°C for 30 min. After incubation, the beads and supernatants were separated by centrifugation and subjected to immunoblotting analyses.

Supplemental Data

Supplemental Data include four figures and one table can be found with this article online at http://www.cell.com/cgi/content/full/127/1/139/DC1/.

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