Phosphorylation of proliferating cell nuclear antigen promotes cancer progression by activating the ATM/Akt/GSK3β/Snail signaling pathway

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Proliferating cell nuclear antigen (PCNA) and its posttranslational modifications regulate DNA metabolic reactions, including DNA replication and repair, at replication forks. PCNA phosphorylation at Tyr-211 (PCNA-Y211p) inhibits DNA mismatch repair and induces misincorporation during DNA synthesis. Here, we describe an unexpected role of PCNA-Y211p in cancer promotion and development. Cells expressing phosphorylation-mimicking PCNA, PCNA-Y211D, show elevated hallmarks specific to the epithelial-mesenchymal transition (EMT), including the up-regulation of the EMT-promoting factor Snail and the down-regulation of EMT-inhibitory factors E-cadherin and GSK3β. The PCNA-Y211D–expressing cells also exhibited active cell migration and underwent G2/M arrest. Interestingly, all of these EMT-associated activities required the activation of ATM and Akt kinases, as inactivating these protein kinases by gene knockdown or inhibitors blocked EMT-associated signaling and cell migration. We concluded that PCNA phosphorylation promotes cancer progression via the ATM/Akt/GSK3β/Snail signaling pathway. In conclusion, this study identifies a novel PCNA function and reveals the molecular basis of phosphorylated PCNA-mediated cancer development and progression.

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2 The abbreviations used are: PCNA, proliferating cell nuclear antigen; EGFR, epidermal growth factor receptor; EMT, epithelial-mesenchymal transition; DEGs, differentially expressed genes; GSEA, gene set enrichment analysis; IPA, Ingenuity Pathway Analysis; ATM, ataxia telangiectasia, mutated; GSK3β, glycogen synthase kinase 3 β; DOX, doxycycline.

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Phosphorylated PCNA stimulates cancer progression

Figure 1. Phosphorylated PCNA promotes cell migration. A, schematic diagram showing the knocking-in (KI) locus at AAVS1 and the composition of the inducible FLAG-tagged PCNA gene, whose expression is controlled by the Tet-On 3G system containing the CAG constitutive synthetic promoter (CAG Pr), the Tet-On 3G transactivator protein (Tet-on 3G), the third generation of the tetracycline response element (TRE3G), the self-cleaving F2A peptide (F2A), and the woodchuck hepatitis virus posttranscriptional regulatory element (WPRE). B and C, Western blot analysis demonstrating expression of the individual forms of PCNA using an antibody against PCNA (B) and the FLAG tag (C). D, GSEA of the DEG in PCNA_YD cells, which reveals the enrichment of the EMT hallmarks. The x axis shows the rank order of genes, from the most up-regulated to the most down-regulated, between HeLa-PCNA_YD and HeLa-PCNA_WT cells. The barcode indicates the position of related genes in the ranking list. The y axis shows the distribution of the running enrichment score generated by walking down the list of ranked genes. E and F, transwell cell migration assay showing statistical analysis of the assay results (E) and representative images of migrated cells after crystal violet staining (scale bar, 100 μm) (F). The results are expressed as the mean ± S.D. of three independent experiments; ns, not significant. ****, p < 0.0001.

Up-regulation of Snail is associated with phosphorylated PCNA-mediated EMT

Transcription factor Snail is an important driving factor of EMT, as it represses the expression of E-cadherin, a transmembrane glycoprotein that connects epithelial cells together at adherens junctions to prevent EMT (35–38). To determine if Snail and E-cadherin are involved in PCNA_YD–promoted EMT, we performed Western blotting experiments and showed that the protein level of Snail in PCNA_YD cells is at least 41% more than in nonphosphorylated control cells (Fig. 2A). Also, little E-cadherin was detected in PCNA_YD cells, but the protein was relatively abundant in all three control cell lines (Fig. 2A). To determine whether the up-regulated Snail is responsible for the observed EMT in PCNA_YD cells, we knocked down Snail expression using an shRNA. As expected, partial depletion of Snail was associated with increased expression of E-cadherin (Fig. 2B). The transwell migration assay revealed that Snail knockdown dramatically reduced the cell migration capability of PCNA_YD cells (Fig. 2, C and D). We, therefore, conclude that phosphorylated PCNA promotes EMT via up-regulating transcription factor Snail.

Up-regulation of Snail in PCNA_YD cells is mediated through the PI3K/Akt pathway

To determine the molecular basis by which Snail is up-regulated in HeLa-PCNA_YD cells, we conducted Ingenuity Pathway...
Analysis (IPA) of the DEG profile, as described (39, 40). Among the top seven canonical pathways identified that are unique to PCNA\(_{\text{YD}}\) cells, as opposed to PCNA\(_{\text{WT}}\) cells, we found that the phosphatidylinositol 3-kinase (PI3K)/Akt pathway is the most active one, with a positive Z-score of 1.81 (Fig. 3A), suggesting that the PI3K/Akt pathway is associated with PCNA-YD–mediated EMT. We also compared the IPA data in PCNA\(_{\text{YD}}\) cells with those in HeLa and HeLa-PCNA\(_{\text{YF}}\) cells and plotted the enrichment scores as a heat map in Fig. 3B. The results showed that activation of the PI3K/Akt signaling pathway was the most enriched.

ATM (ataxia telangiectasia, mutated), a PI3K-related kinase, has been shown to promote cancer metastasis via stabilizing Snail (41). It is also known that the PI3K/Akt pathway downregulates glycogen synthase kinase 3 \(\beta\) (GSK3\(\beta\)) and up-regulates Snail (42–46). We hypothesized that these molecules (ATM, Akt, GSK-3\(\beta\), and Snail) lie in the phosphorylated PCNA-mediated EMT signaling pathway. Upon inducing PCNA molecules by doxycycline (DOX), we analyzed cell lysates from various PCNA-expressing cells for activation of ATM and Akt, as well as up- and down-regulation of Snail and GSK3\(\beta\), respectively. As shown in Fig. 3C, PCNA\(_{\text{YD}}\) cells, but not the three control cell lines (HeLa, PCNA\(_{\text{WT}}\), and PCNA\(_{\text{YF}}\)), expressed a high level of Ser-1981–phosphorylated ATM (active form). Increased phosphorylation of both Akt (Ser-473, active form) and GSK3\(\beta\) (Ser-9, inactive form) was also observed in cells expressing PCNA-YD, but not in the three control cell lines (Fig. 3C). Accordingly, high levels of Snail were associated with these phenomena (Fig. 3C). To verify the involvement of ATM signaling in PCNA-YD–promoted EMT, we treated cells with wortmannin, a PI3K-Akt pathway inhibitor (47–49). We found that wortmannin treatment dramatically reduced the phosphorylation levels of ATM, Akt, and GSK3\(\beta\) (Fig. 3D). We also observed a dramatic reduction in Snail expression (Fig. 3D). In contrast, the treatment led to an increase in E-cadherin expression (Fig. 3D). Consistent with these anti-EMT phenomena, the transwell migration assay showed that wortmannin treatment blocked PCNA\(_{\text{YD}}\) cells’ ability to migrate (Fig. 3, E and F). These results strongly suggest that the EMT in PCNA\(_{\text{YD}}\) cells is processed through the PI3K/Akt/GSK3\(\beta\)/Snail signaling axis.

We examined this idea further with experiments that directly inactivated ATM. First, we knocked down the expression of ATM in HeLa-PCNA\(_{\text{YD}}\) cells using shRNAs against ATM and measured their EMT-related activities. The results showed a 45% reduction in ATM (Fig. 4A). This partial reduction in ATM essentially blocked Akt activation. As expected, the knockdown was also associated with increased expression of GSK3\(\beta\) and decreased expression of Snail (Fig. 4A). More importantly, the partial depletion of ATM substantially reduced PCNA\(_{\text{YD}}\) cells’ migration capacity (Fig. 4, B and C). Second, we treated HeLa-PCNA\(_{\text{YD}}\) cells with KU-55933, an ATM-specific inhibitor. As expected, KU-55933 treatment inactivated ATM and blocked the downstream EMT process, much like what we observed in ATM knockdown cells (Fig. 4, D–F). We, therefore, conclude that the ATM/Akt/GSK3\(\beta\)/Snail signaling axis executes phosphorylated PCNA-mediated EMT.
Cells expressing PCNA-YD arrest at G₂/M

It is well-documented that EMT is associated with G₂/M cell cycle arrest (50), although the cause and effect relationship is unclear. We therefore analyzed cell cycle distribution in cells expressing individual forms of PCNA. The results showed that all three control cell lines (HeLa, HeLa-PCNA_WT, and HeLa-PCNA_YD) demonstrated essentially the same pattern of cell cycle phases, but the pattern in PCNA_YD cells is visibly different from that of the control cells (Fig. 5, A and B), as PCNA_YD cells show more than twice (35% versus 17%) as many G₂/M cells as each of the control cell lines (Fig. 5C), suggesting a G₂/M arrest in PCNA_YD cells. Morphologically, many PCNA_YD cells exhibit a much larger nucleus (see red arrows) than control cells in Hoechst stain (Fig. 5D), indicating abnormal mitotic division in PCNA_YD cells. Consistent with this assumption, PCNA_YD cells contained a higher percentage of cells with a DNA content greater than 4N, compared with control cells. The production of these abnormal nuclei appears to depend on phosphorylated PCNA, because the percentage of cells with an abnormal nucleus is proportional to the length of DOX treatment (Fig. 5E). We then analyzed Cdc2 and Cdc25C, both of which are hallmarks of G₂/M arrest (51–53). We observed high levels of phosphorylated Cdc2 (Tyr-15) and phosphorylated Cdc25C (Ser-216) in PCNA_YD cells (Fig. 5F). Correspondingly, we detected phosphorylation of Chk1 and Chk2, downstream substrates of activated ATR and ATM, respectively, and upstream kinases of Cdc25C, in PCNA_YD cells. These results indicate that PCNA phosphorylation–induced EMT is associated with G₂/M arrest, which is probably triggered through the ATM/ATR signaling pathway (51–53). However, given the fact many PCNA_YD cells underwent nuclear degradation when treated with an ATR inhibitor (Fig. S1), we believe that ATR activation in HeLa-PCNA_YD cells is unrelated to EMT, but it is essential for cell survival and other cellular functions (54, 55).

Discussion

PCNA is a critical cell proliferation factor that orchestrates essentially all metabolic reactions at the replication fork, including DNA replication and DNA repair (1–3). PCNA can be phosphorylated at Tyr-211 by tumor-promoting factor EGFR (9). We have shown previously that Tyr-211–phosphorylated PCNA inhibits DNA mismatch repair and induces nucleotide misincorporation during DNA synthesis, thereby induc-
ing a mutator phenotype (14). Interestingly, like EGFR, PCNA is also used as a diagnostic and prognostic marker for tumors (17–22), but the underlying mechanism of tumor progression is unclear. In this study, we provide evidence that Tyr-211–phosphorylated PCNA promotes EMT.

We revealed the involvement of phosphorylated PCNA in EMT through a gene set enrichment analysis of RNA-Seq data derived from cells expressing individual isoforms of PCNA examined in this study, which identified molecular hallmarks specific to EMT in PCNA_YD cells (Fig. 1D). Consistent with this prediction, we found that PCNA_YD cells indeed display EMT characteristics. First, these cells exhibit a migration activity that is much more active than that of control cells (Fig. 1, E and F); second, EMT-promoting factor Snail and EMT-inhibitory factor E-cadherin are up- and down-regulated, respectively (Fig. 2A), in PCNA_YD cells; and third, Snail knockdown prevents PCNA_YD cells from migrating (Fig. 2, C and D). We show that Snail-mediated EMT appears to be activated by the PI3K/Akt signaling pathway. Evidence supporting this notion initially came from the IPA of the DEG’s profile, where the PI3K/Akt signaling pathway shows the most active one in PCNA_YD cells (Fig. 3C).

The activation of ATM and Akt is coupled with the down-regulation of GSK3β and the up-regulation of Snail (Fig. 3C). Inhibition of the ATM/Akt signaling pathway by wortmannin, KU-55933, or ATM knockdown blocks the up-regulation and down-regulation of Snail and GSK3β (Figs. 3D and 4, A and D), respectively, as well as the migration activity of PCNA_YD cells (Figs. 3E and 4, B and E).

On the basis of previously published data and the results presented here, we propose a signaling cascade for PCNA phosphorylation–induced EMT (Fig. 6). It has been shown that phosphorylated PCNA inhibits DNA mismatch repair and induces misincorporation during DNA synthesis (14); cells with phosphorylated PCNA make numerous errors during replication. Even though the molecular basis underlying phosphorylated PCNA–induced misincorporation is unclear, it has been postulated that the modified PCNA recruits an error-prone translesion polymerase to carry out DNA synthesis. The nonprocessive nature of translesion polymerases can lead to a severe delay in DNA replication. Although the delay in replication induces G2/M arrest, both the replication errors and the delays cause replication stress to activate the ATM-mediated DNA damage response. The activated ATM, which also facilitates G2/M arrest (51), then triggers a cascade of signaling reactions, leading to cell cycle arrest.
which mediate phosphorylations of Akt, GSK3β, and Snail to promote EMT (41).

However, there are many uncertainties. Although activation of the ATM/ATR DNA damage response pathway can trigger G2/M cell cycle arrest (51), recent studies also suggest that the process of EMT induces G2/M arrest (49). It is well-established that genomic instability can lead to polyploidy/aneuploidy, which can result from G2/M arrest and abnormal cell division, leading to cancer development and progression (56). Thus, whether the G2/M arrest observed in HeLa-PCNA_{YD} cells is induced by replication stress–activated ATM signaling or is a result of EMT is unknown. It is possible that these processes mutually promote each other. Another big question concerns the exact abnormal event or signal generated by the phosphorylated PCNA–mediated reaction at the replication fork that triggers the ATM/Akt signaling pathway to promote EMT. Future studies are required to address these important questions.

**Experimental procedures**

**Cell culture and materials**

HeLa and HeLa knock-in cell lines were cultured in DMEM with 10% FBS at 37 °C in a humidified atmosphere with 5% (v/v) CO2. To induce the expression of the knock-in PCNA, cells were treated with 1.0 μg/ml of DOX for 4 days. When present, the concentration of KU55933 (Selleck, S1092) or wortmannin (Selleck, S2758) used was 10 μM or 2 μM, respectively.

The following antibodies and chemicals were purchased commercially: anti-PCNA (Santa Cruz Biotechnology, sc-56); anti-β-tubulin (Bioeasy, BE0031); anti-FLAG tag (Bioeasy, BE2004); anti-E-cadherin (Santa Cruz Biotechnology, sc-8426); anti-Snail (Cell Signaling Technology, 3895); anti-Akt (Cell Signaling Technology, 9272); anti-phosphorylated Akt Ser-473 (Cell Signaling Technology, 4060); anti-GSK3β (Cell Signaling Technology, 9315); anti-phosphorylated GSK3β Ser-9 (Cell Signaling Technology, 9272); anti-phosphorylated Akt Ser-473 (Cell Signaling Technology, 4060); anti-GSK3β (Cell Signaling Technology, 9272); anti-phosphorylated GSK3β Ser-9 (Cell Signaling Technology, 9272); anti-phosphorylated GSK3β Ser-9 (Cell Signaling Technology, 9272); anti-phosphorylated GSK3β Ser-9 (Cell Signaling Technology, 9272); anti-phosphorylated GSK3β Ser-9 (Cell Signaling Technology, 9272); anti-phosphorylated GSK3β Ser-9 (Cell Signaling Technology, 9272); anti-phosphorylated GSK3β Ser-9 (Cell Signaling Technology, 9272); anti-phosphorylated GSK3β Ser-9 (Cell Signaling Technology, 9272); anti-phosphorylated GSK3β Ser-9 (Cell Signaling Technology, 9272); anti-phosphorylated GSK3β Ser-9 (Cell Signaling Technology, 9272); anti-phosphorylated GSK3β Ser-9 (Cell Signaling Technology, 9272); anti-phosphorylated Cdc25c Ser-216 (Abcam, ab47322); and anti-phosphorylated cdc2 Tyr-15 (Cell Signaling Technology, 4539).

**Cell cycle analysis by flow cytometry**

For cell cycle determination, cells were cultured in media with 10 μg/ml Hoechst for 10 min before harvesting. Cells were then fixed with 75% ethanol before cytometry analysis.
**In vitro transwell cell migration assay**

An *in vitro* migration assay was performed using a 24-well Boyden chamber (32). Approximately 5 × 10^4 cells (in 100 µl media) were treated with DOX for 4 days and added to the upper chamber in serum-free medium containing DOX. The lower compartment was filled with 650 µl medium containing 10% FBS and DOX. All cells were seeded in the upper part of the Boyden chamber and incubated for 12 h. Nonmigrated cells were scraped from the upper surface of the membrane with a cotton swab. Migrated cells remaining on the bottom surface were stained with 0.5% crystal violet for 20 min. The migratory phenotypes were determined by counting the cells that migrated to the lower side of the filter using microscopy at 10 × magnification. Five fields were counted for each filter, and each sample was assayed in triplicate.

**shRNA experiment**

shRNAs for ATM and Snail were synthesized at the Center for Biomedical Analysis at Tsinghua University and cloned into the pLKO.1 vector. A scrambled shRNA was used as a control, whose sequence is CGGGCAAGAATGGAAGAGCACAACCTCGAGTTGGTGCTCCTCATTTGTTTTTTT. Five different shRNAs were used for each shRNA knockdown. Western blot analysis was used to determine protein knockdown. We then selected the two most effective knockdown clones. For shSnail, effective knockdown was from sequences CCGGGCAACGCAAATCTCAGTGATATCTCGAGAACTCTGGATTAGAGTCCTGCTTTTTG and CCGGGCAGGACTCTAATCCAGAGTTGGTGCTCTTCATCTTGTTT. For shATM, these sequences were CCGGCAAACGAAATCTCAGTGATATCTCGAGAACTCTGGATTAGAGTCCTGCTTTTTG. For shPCNA, these sequences were CCGGCAAACGAAATCTCAGTGATATCTCGAGAACTCTGGATTAGAGTCCTGCTTTTTG. For shSnail, these sequences were CCGGCAAACGAAATCTCAGTGATATCTCGAGAACTCTGGATTAGAGTCCTGCTTTTTG. For shPCNA, these sequences were CCGGCAAACGAAATCTCAGTGATATCTCGAGAACTCTGGATTAGAGTCCTGCTTTTTG.

**RNA-Seq and analysis**

RNAs were isolated from cells treated with DOX for 4 days using TRIzol Reagent (Invitrogen), and sequencing libraries were generated using NEBNext Ultra™ RNA Library Prep Kit for Illumina® (New England Biolabs). The resulting libraries were sequenced on an Illumina platform and 150-bp pair-length reads were collected. Differentially expressed genes of two groups (HeLa-PCNAYD versus HeLa, HeLa-PCNAYF, or HeLa-PCNAWT, or HeLa-PCNAVP) were calculated using software DESeq (1.12.0). The resulting *p* values were adjusted using the Benjamini-Hochberg procedure. Genes with an adjusted *p* value <0.05 were assigned as differentially expressed. The complete unedited RNA-Seq datasets are available at Gene Expression Omnibus (GEO) database (accession number GSE127276).

The differentially expressed genes were used for GSEA analysis (30, 31). The gene set collection used was the h.all.v6.2_symbols.gmt [Hallmarks] gene sets database. GSEA analyzed 1000 permutations, and enrichment statistic was weighted. For IPA, the differentially expressed coding genes identified by the RNA-Seq at a *p* <0.05, -fold change >2 were uploaded to the IPA software for canonical pathway analysis. The analysis was conducted based on prior knowledge of pathway network stored in the Ingenuity Knowledge Database. The *p* value represents the significance of a given pathway and the Z-score predicts the pathway activation or inactivation.

**Western blot analysis**

Whole cell lysates were obtained in the radioimmunoprecipitation assay lysis buffer containing protease and phosphatase inhibitors. Protein concentrations were determined using BCA Protein Assay Reagents (Thermo Fisher). Proteins were separated by SDS-PAGE on polyacrylamide gels, transferred onto PVDF membranes, and detected by Western blot analysis using antibodies against specific proteins.

**Statistical analysis**

All statistical analyses were performed with one-way analysis of variance (ANOVA) test using GraphPad Software. Data were expressed as mean ± S.D. and were considered statistically significant if *p* values were less than 0.05 or 0.01, as indicated.

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