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# Mammalian PER2 Regulates AKT Activation and DNA Damage Response

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**Abstract:** PER2 is a key mammalian circadian clock protein. It also has a tumor suppressive function. Down regulation of *PER2* in the cultured cancer cells accelerates cell proliferation, while overexpression of PER2 inhibits cell growth and induces apoptosis. The *Per2* mutant mice have a cancer prone phenotype and an altered DNA damage response. Here we report that PER2 regulates AKT activity. Cells with down-regulated *PER2* expression have prolonged high levels of AKT T308 phosphorylation after growth factor stimulation or DNA damage. *PER2* down-regulation delays DNA damage induced Chk2 activation and overrides DNA damage induced apoptosis and cell cycle arrest.

*Key words:* PER2, AKT, tumor suppressor, DNA damage response.

**Résumé :** PER2 est une protéine clé de l'horloge circadienne des mammifères. Elle possède également une fonction suppressive des tumeurs. La régulation à la baisse de *PER2* chez des cellules cancéreuses en culture accélère la prolifération alors qu'une surexpression de PER2 inhibe la croissance cellulaire et induit l'apoptose. Des souris *Per2* mutantes possèdent un phénotype de susceptibilité au cancer et une réponse déficiente aux dommages à l'ADN. Nous rapportons ici que PER2 régule l'activité d'AKT. Le haut niveau de phosphorylation d'AKT T308 se maintient plus longtemps après une stimulation par facteur de croissance ou des dommages à l'ADN chez les cellules où l'expression de *PER2* est régulée à la baisse. La régulation à la baisse de *PER2* retarde l'activation de Chk2 induite par les dommages à l'ADN, et annule l'apoptose et l'arrêt du cycle cellulaire induits par les dommages à l'ADN.

*Mots-clés :* PER2, AKT, suppresseur de tumeur, réponse au dommage à l'AND.

[Traduit par la Rédaction]

## Introduction

In the mammalian clockwork, the *Period* genes (*Per1, 2*) are among the most critical clock genes (Reppert and Weaver 2002). In addition to their roles in controlling mammalian circadian rhythms, several lines of evidence suggest that the *Period* genes also have unique tumor suppressive functions. For example, *Per2* mutant mice not only have a disrupted circadian clock but also show deregulated expressions of cell cycle control genes (*c-Myc*, *Cyclin D1*, *Gadd45*, *Mdm2*) and an abnormal DNA damage response, thus leading to a cancer prone phenotype (Fu et al. 2002). In human tumor tissues, *PER1* and *PER2* are often deregulated. Decreased *PER2* RNA levels have been reported in acute leukemias and in breast tumors (Gery et al. 2005; Winter et al. 2007). Abnormalities in *PER2* (and *PER1*) promoter methylation and asynchronous protein expression have also been observed in breast tumors (Chen et al. 2005). Furthermore, it has been reported that a decreased level of *PER2* in colorectal tumors predicts a poor survival (Iacobelli et al. 2008; Oshima et al. 2011; Zeman et al. 2008).

We have shown that *Per2* mutant mice develop colonic polyps and have increased expressions of β-catenin and its downstream genes (Wood et al. 2008). In addition, introduction of *Per2* mutation into $Apc^{Min/+}$ mice enhances $Apc^{Min/+}$ associated phenotypes, including increased intestinal polyp number, anemia, and increased spleen weight (Wood et al. 2008). We have also shown that down-regulation of *PER2* increases the growth of cancer cells under normal growth conditions (Wood et al. 2008; Yang et al. 2009). Others have shown that over expression of PER2 inhibits cell proliferation and enhances apoptosis in both cultured cancer cells and transplanted tumors (Gery and Koeffler 2009; Hua et al. 2006, 2007). However, it is unclear how PER2 exerts its tumor suppressive function.

AKT is a key regulator of β-catenin expression and cell proliferation (Liang and Slingerland 2003; Liao and Hung 2010). AKT inhibits apoptosis and promotes cell growth, and its overactivation has been implicated in several types of cancer (Cicenas 2008). After growth factor stimulation, PI3K generates messenger molecule PIP3, which recruits AKT to the cell membrane where it is phosphorylated at the T308 site by PDK1 and at the S473 site by mTORC2. The activated AKT phosphorylates its substrates, such as GSK3β at the S9 site, and this inactivates GSK3β (MacDonald et al. 2009). AKT is also phosphorylated by DNA-PK after DNA

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**Fig. 1.** *PER2* deficiency enhances AKT activation after growth factor stimulation. After HCT116 cells were treated with siRNA (Luciferase siRNA as control and *PER2* siRNA set 1) for 48 h, 100ng/mL of IGF-1 was added to the medium. Samples were taken at the indicated time points. The relative amounts of *PER2* mRNA were determined by real time PCR. The amount in the control cells at time point 0 was set as 1 (A). The amounts of phosphorylated AKT and GSK3β, and total level of PTEN, were examined by specific antibodies. GAPDH was the loading control (B). Quantification of AKT T308, S473, and GSK3β S9 in the control (gray bars) and *PER2* knockdown (empty bars) cells after IGF-1 stimulation. The levels before the treatment in control cells were set as 1 (C). *PER2* was down-regulated by *PER2* siRNA set 2 in HCT116 cells. Cells were treated by 100ng/mL of IGF-1 for the indicated time. The relative amounts of *PER2* mRNA were determined by real time PCR. The amount in the control cells at time point 0 was set as 1 (D). The phosphorylation levels of AKT T308, S473, and GSK3β S9 were determined by Western blotting (E). Primary MEF cells were isolated from wild type or $Per2^{m/m}$ mice. The genotype was determined by PCR according to the protocol provided by Jackson Laboratory cells were treated with 100ng/mL of IGF-1 for the indicated time. The T308 phosphorylated and total AKT were compared (F).



damage (Bozulic et al. 2008). However, AKT overactivation leads to the inhibition of DNA damage response (Xu et al. 2010). Here we report that PER2 may exert its tumor suppressive function by regulating AKT activity. We demonstrate that PER2-depleted cells have a higher level of phosphorylated AKT. Furthermore, down-regulation of *PER2* leads to a prolonged AKT activation after growth factor stimulation or DNA damage. Enhanced AKT activity in *PER2* down-regulated cells attenuates doxorubicin induced DNA damage response and renders cells resistant to chemotherapy drugs.

## Materials and methods

### Cell Culture and siRNA

HCT116 and SW480 human colon cancer cells obtained from ATCC (Manassas, Va., USA) were maintained in RPMI 1640 medium with 10% fetal bovine serum (FBS) under the condition of 5% $CO_2$ at 37 °C. For siRNA transfection, cells were plated in 6-well plates the day before to reach 30% confluency by the time of transfection. siRNA oligos (200 pmol) were transfected by Lipofectmine2000 (Invitrogen, Carlsbad, Calif., USA) according to the protocol provided by the manufacturer. The knockdown efficiency was examined after 48–72h of incubation by real-time PCR. The sequences of human *PER2* siRNA were designed by Blockit siRNA Designer (Invitrogene). Sequences of RNA oligos were as follows:

1. Control (Luciferase): 5′-CGUAACGCGGAAUACUUCGAdTdT-3′ and 5′-UCGAAGUAUUCCGCGUACGdTdT-3′
2. *PER2* (Set 1): 5′-GCGUUACCUCUGAGCACAUdTdT-3′ and 5′-AUGUGCUCAGAGGUAACGCdTdT-3′
3. *PER2* (Set 2): GCGCUAAGGUCCAGUGAUAdTdT-3′ and 5′-UAUCACUGGACCUUAGCGCdTdT-3′

The sequences for real time PCR quantification of human *PER2* were:

1. *PER2* Forward: 5′-CCTCTCCTGGGCTACCTACC-3′ and
2. *PER2* Reverse: 5′-CCTCCCAATGATGAAGGAGA-3′

*GAPDH* was used as a control for real time PCR. The sequences for *GAPDH* were:

1. *GAPDH* Forward: 5′-GAGTCAACGGATTTGGTCGT-3′ and
2. *GAPDH* Reverse: 5′-TTGATTTTGGAGGGATCTCG-3′

MEF cells were generated from the wild type C57BL/6J and $Per2^{m/m}$ mice (Jackson laboratory, Bar Harbor, Maine, USA). $Per2^{m/m}$ mice had been backcrossed into C57BL/6J background for 10 generations. MEF cells were cultured in DMEM with 10% FBS.

### Western blot and antibodies

After 48 h of siRNA transfection, cells were treated with doxorubicin (Sigma, St. Louis, Mo., USA) for the indicated time, or treated for 2 h then incubated in the drug free medium. Cells were washed with PBS once, and total protein extracts were obtained by incubating in NP-40 buffer (0.5% NP-40, 150 mmol/L NaCl, 50 mmol/L Tris-Cl pH7.4, protease inhibitors). Proteins were then separated by standard SDS–PAGE and transferred to nitrocellulose membranes. Antibodies against Chk2 T68, AKT T308, AKT S473, GSK3β S9, and γ–H2AX were from Cell Signaling Technology (Danvers, Mass., USA). Anti-PER2 antibodies were from Alpha Diagnostic International (San Antonio, Tex., USA). Other antibodies were from Santa Cruz Biotechnology (Santa Cruz, Calif., USA). AKT1/2 inhibitor was from Sigma and dissolved in DMSO. All experiments were repeated at least 3 times. Images were quantified by Image J software (NIH, USA). Statistic difference was determined by *t* test and annotated with an asterisk.

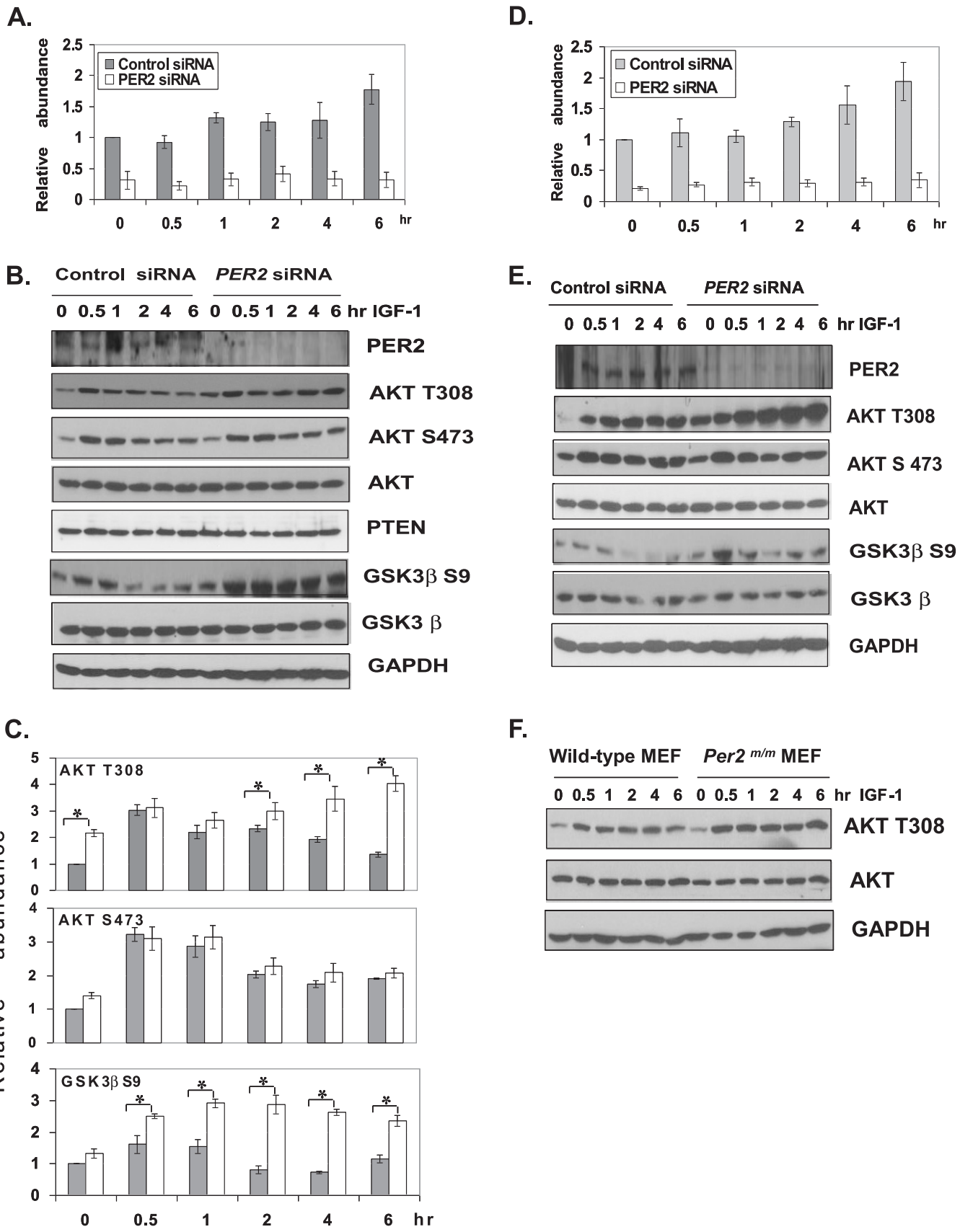
### Drug sensitivity assay

After siRNA treatment for 48 h, cells were incubated with the indicated concentration of drug for 24 h. Then cells were washed with PBS, trypsinized, and seeded in the drug-free medium. Cells were fixed with methanol and stained with crystal violet solution after 1 week of incubation. Colonies were counted.

## Results

### PER2 deficient cells have enhanced AKT activity

The increased proliferation of *PER2* down-regulated cells prompted us to further investigate the underlying mechanism. It has been reported that the endothelial cells of *Per2* mutant mice exhibit AKT-dependent senescence (Wang et al. 2008). Since we have observed that *Per2* mutation enhances intestinal tumorigenesis in mice, we used colorectal cancer cells to investigate the role of PER2 in vitro. Down-regulation of *PER2* in HCT116 cells increased the basal level of T308 phosphorylated AKT (Figs. 1 A and 1B). IGF-1, a growth factor, acutely stimulated AKT phosphorylation at both T308 and S473 sites (Figs. 1B and 1C). AKT phosphorylations gradually reduced over the time in the control cells. However, *PER2* knockdown cells had prolonged high levels of AKT T308 phosphorylation, while the levels of S473 phosphorylated AKT did not differ between the control and *PER2*



knockdown cells. To determine whether prolonged AKT T308 phosphorylation caused AKT over-activation, the phosphorylation level of GSK3β at the S9 site was examined. The phosphorylated GSK3β also remained high in *PER2* knockdown cells, suggesting that growth factor induced AKT was over-activated in *PER2* knockdown cells. AKT T308 is phosphorylated by PDK1. The total amount of PDK1 protein did not change in *PER2* down-regulated cells (data not shown). The expression of PTEN, a negative regulator of PDK1, did not differ in the control and *PER2* down-regulated cells either (Fig. 1B). We used another set of *PER2* siRNA to knockdown *PER2* in HCT116 cells (Fig. 1D). Again, in *PER2* down-regulated cells, the phosphorylation levels of AKT T308 and GSK3β S9 were enhanced (Fig. 1E). IGF-1 stimulated AKT phosphorylation was also examined in the wild type and $Per2^{m/m}$ MEF cells. $Per2^{m/m}$ MEF cells had a prolonged AKT T308 phosphorylation after IGF-1 treatment (Fig. 1F).

AKT is also an important player in DNA damage response. DNA damage temporarily induces AKT phosphorylation and activation. This process is dependent on mismatch repair (MMR) genes (Caporali et al. 2008). We tested the kinetics of AKT phosphorylation in HCT116 cells after DNA double strand breaks were induced by doxorubicin (Dox) treatment. HCT116 is an MMR deficient cell line, therefore radiation-induced DNA damage does not cause AKT phosphorylation in HCT116 cells (Xu et al. 2010). When HCT116 cells were treated with Dox, the level of AKT T308 phosphorylation in the control cells decreased but remained high in the *PER2* down-regulated cells (Figs. 2A–2C). In fact, AKT T308 phosphorlation slightly increased after Dox treatment in the *PER2* knockdown cells. There was no significant difference in AKT S473 levels between the control and *PER2* knockdown cells (Figs. 2B and 2C). We also examined AKT phosphorylation in SW480 cells. SW480 is an MMR proficient human colon cancer cell line. As expected, AKT phosphorylation was increased at both T308 and S473 sites after Dox treatment and their levels were higher in *PER2* knockdown cells (Figs. 2D–2F). After 20 h, AKT S473 phosphorylation dropped to the basal level in both the control *PER2* knockdown cells. AKT T308 phosphorylation in the control cells dropped after 20 h of Dox treatment but remained elevated in *PER2* knockdown cells (Figs. 2E and 2F).

### *PER2* down-regulation attenuates DNA damage response

To determine whether *PER2* down-regulation had an effect on the DNA damage response, HCT116 cells were treated with a low dose of Dox (0.5 μmol/L) for 2 h to induce DNA double strand breaks (DSBs). In the control cells, Chk2 was quickly phosphorylated at T68 site, and the Chk2 T68 level was reduced after 24 h (Figs. 3A and 3B). However, the DSB-induced Chk2 activation was significantly delayed in *PER2* knockdown cells (Fig. 3B). Proper Chk2 T68 phosphorylation is required for subsequent cell cycle arrest and DNA damage repair after DSB. In *PER2* knockdown cells, the accumulation of WEE1, a marker of G2/M cell cycle arrest, was inhibited, suggesting that DNA damage-induced cell cycle arrest was inhibited. Lower levels of cleaved caspase3 in *PER2* down-regulated cells indicated that DNA damage-induced apoptosis was attenuated (Figs. 3B and 3C). We also treated cells with a prolonged Dox exposure (20 h) (Figs. 3D and 3E). PER2 depleted cells had a higher level of phosphorylated Chk2 after a prolonged incubation with Dox (Fig. 3E). The protein levels of WEE1, CycB1, and p53 were lower in *PER2* knockdown cells, indicating that diminished PER2 expression overrode DNA damage-induced cell cycle arrest (Fig. 3E).

To determine whether the delayed Chk2 T68 phosphorylation in *PER2* knockdown cells was dependent on AKT, cells were treated with AKT1/2 inhibitor for 1 h before the treatment with Dox. Inhibition of AKT activity abolished the *PER2* knockdown effect on Chk2 phosphorylation (Fig. 3G). There was no difference in Dox induced γ-H2AX accumulation in the control and *PER2* down-regulated cells, suggesting the absence of PER2 did not affect the cells' ability to sense the DNA damage (Fig. 3G).

### *PER2* down-regulated cells are resistant to chemotherapy drugs

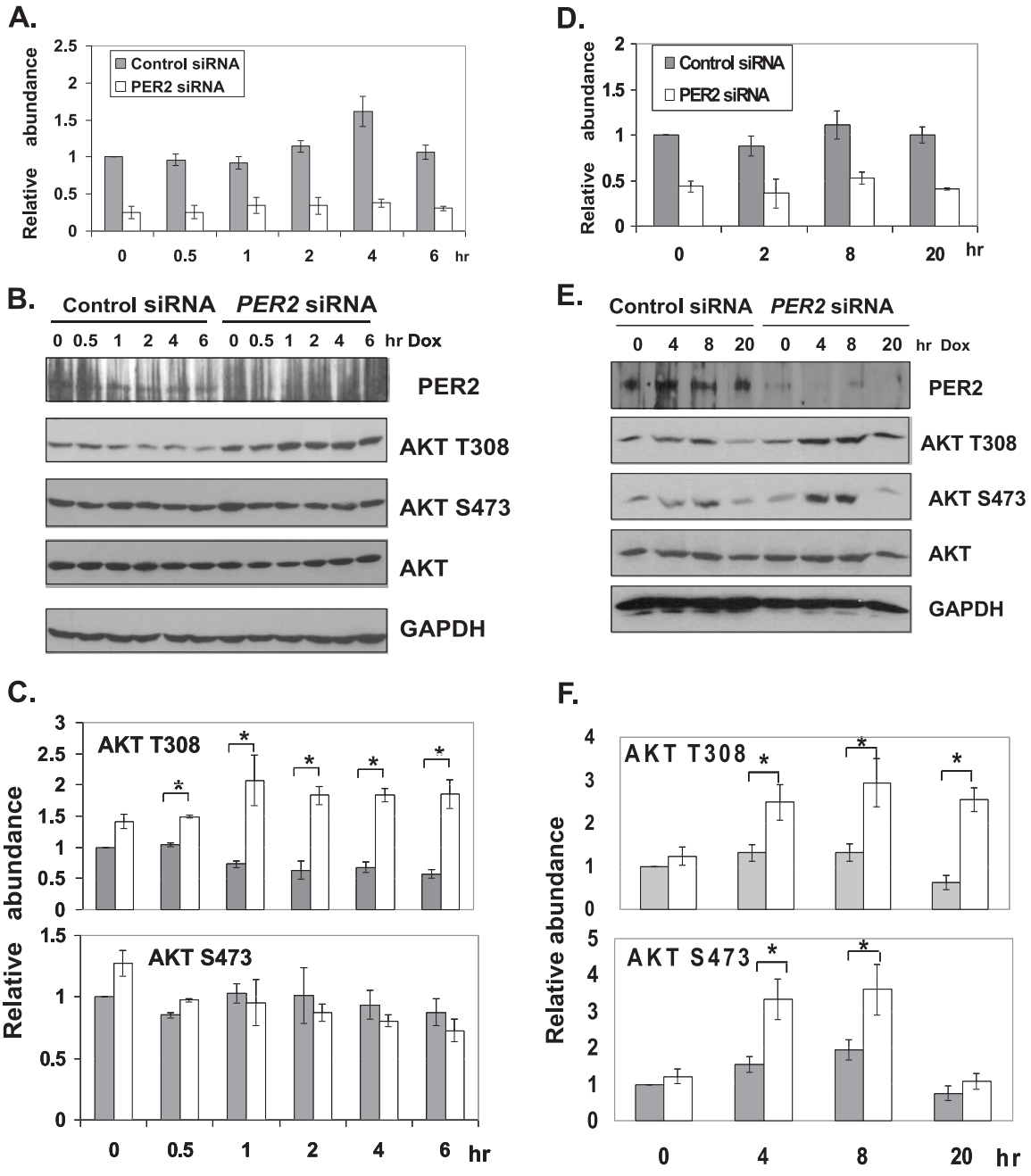
Since *PER2* modulated DNA damage response, we tested whether PER2 depletion altered drug sensitivity of cancer cells. HCT116 cells with *PER2* knocked down were resistant to DNA damage agents, etoposide, and Dox, compared with the control, as determined by colony formation assay (Fig. 4). On the other hand, others have shown that overexpression of PER2 sensitizes cells to irradiation and chemotherapeutic drugs (Oda et al. 2009). It is known that AKT over-activation causes cancer cells resistant to chemotherapy drugs (Henry et al. 2001; Hirose et al. 2005; Lal et al. 2009). It is likely that the drug resistant phenotype of *PER2* depleted cells might be due to the enhanced AKT activation after DNA damage.

## Discussion

Increasing evidence indicates that the core circadian clock protein, PER2, has a tumor suppressive function. Overexpression of PER2 causes apoptosis in the cultured cancer cells (Gery and Koeffler 2009; Hua et al. 2006, 2007). Since PER2 overexpression disrupts the circadian clock, and expressing a protein to a nonphysiological level may cause unexpected effects, we down-regulated *PER2* in cancer cells in our previous studies and have found that *PER2* down-regulation enhances cancer cell proliferation in vivo and in vitro (Wood et al. 2008; Yang et al. 2009). Here we report that PER2 regulates the activity of AKT. *PER2* knockdown cells have a higher basal level of T308 phosphorylated AKT. It is well-known that AKT promotes cell proliferation. Enhanced AKT activity in PER2 depleted cells may contribute to the increased cell proliferation.

In *PER2* knockdown cells, Chk2 T68 phosphorylation is delayed and WEE1 accumulation is inhibited after Dox treatment. It is known that medium change may synchronize the circadian clock in cultured cells. If Chk2 T68 phosphorylation is controlled by the circadian clock then the delayed Chk2 phosphorylation in *PER2* knockdown cells may be due to the disrupted circadian clock. However we do not think this is the case, because with or without medium change after Dox treatment the results are not altered (medium was changed in Fig. 3B but not in Fig. 3E). In addition, we have determined that Chk2 T68 phosphorylation does not change after serum shock in cultured cells. *Wee1* transcription is

**Fig. 2.** Enhanced AKT phosphorylation after DNA damage in *PER2* down-regulated cells. After siRNA treatment (Luciferase siRNA or *PER2* siRNA set 1), HCT116 cells (A, B, C) and SW480 cells (D, E, F) were treated with 0.5 μmol/L of doxorubicin for the indicated time. The relative amounts of *PER2* mRNA were determined by real time PCR. The amounts in the control cells at time point 0 were set as 1 (A, D). The amounts of phosphorylated AKT and total AKT were determined by specific antibodies (B, E). Quantification of AKT T308 and AKT S473 phosphorylation in HCT116 (C) and SW480 (F) (Control, gray bars; Per2 knockdown, empty bars). The amounts in the control cells before treatment were set as 1.




suppressed by PER/CRY complex (Matsuo et al. 2003). Knockdown of CRY leads to an elevated expression of WEE1 (Gauger and Sancar 2005). However, in *PER2* knockdown cells, WEE1 accumulation is inhibited after Dox treatment. Therefore, it is unlikely that the altered WEE1 expression in *PER2* knockdown cells is due to the disrupted circadian clock.

AKT overactivation is known to override DNA damage-induced cell cycle arrest and apoptosis in various cells including HCT116 (Hirose et al. 2005; Kandel et al. 2002; Xu et al. 2010). Several studies have shown that AKT prevents the binding of DNA damage mediators, such as RAD51 and RPA, from forming foci at the damaged DNA sites (Plo et al. 2008; Xu et al. 2010). Therefore, active AKT interferes the tranduction of DNA damage signal from sensors to effectors. *PER2* knockdown cells can still sense the damaged DNA because γ-H2AX accumulation after DNA damage is not affected by the absence of PER2.

**Fig. 3.** Down-regulation of *PER2* alters DNA damage response. HCT116 cells were treated with luciferase siRNA (control) or *PER2* siRNA set 1 for 48 h followed by 0.5 μmol/L doxorubicin treatment for 2 h. Cells were then incubated in the drug-free medium. Samples were taken at the indicated time points. (NT: no doxorubicin treatment). The relative amounts of *PER2* mRNA were determined by real-time PCR. The amount in the NT control cells was set as 1 (A). Chk2 T68, WEE1, and caspase 3 were detected by specific antibodies. GAPDH was the loading control (B). Quantification of WEE1 and cleaved caspase 3 in the control (gray bars) and *PER2* knockdown cells (empty bars). The amounts in the control cells without drug treatment were set as 1(C). After 48 h of siRNA treatment, HCT116 cells were incubated with 0.5 μmol/L of doxorubicin for 20 h. The relative amounts of *PER2* mRNA were determined by real-time PCR. The amount in control cells at the time point 0 was set as 1 (D). The expression of proteins determined by Western blotting. GAPDH was the loading control (E). Control or *PER2* knockdown HCT116 cells were treated with AKT1/2 inhibitor (20 μmol/L) for 1 h followed by treatment with 2 μmol/L of doxorubicin for 2 h. Cells were then incubated in the drug-free medium for the indicated time (NT, no Dox treatment). The relative amounts of *PER2* mRNA were determined by real time PCR. The amount in the NT control cells without AKTi treatment was set as 1 (F). The levels of Chk2 T68, total Chk2, and γ-H2AX were determined by Western blotting. GAPDH was the loading control (G).

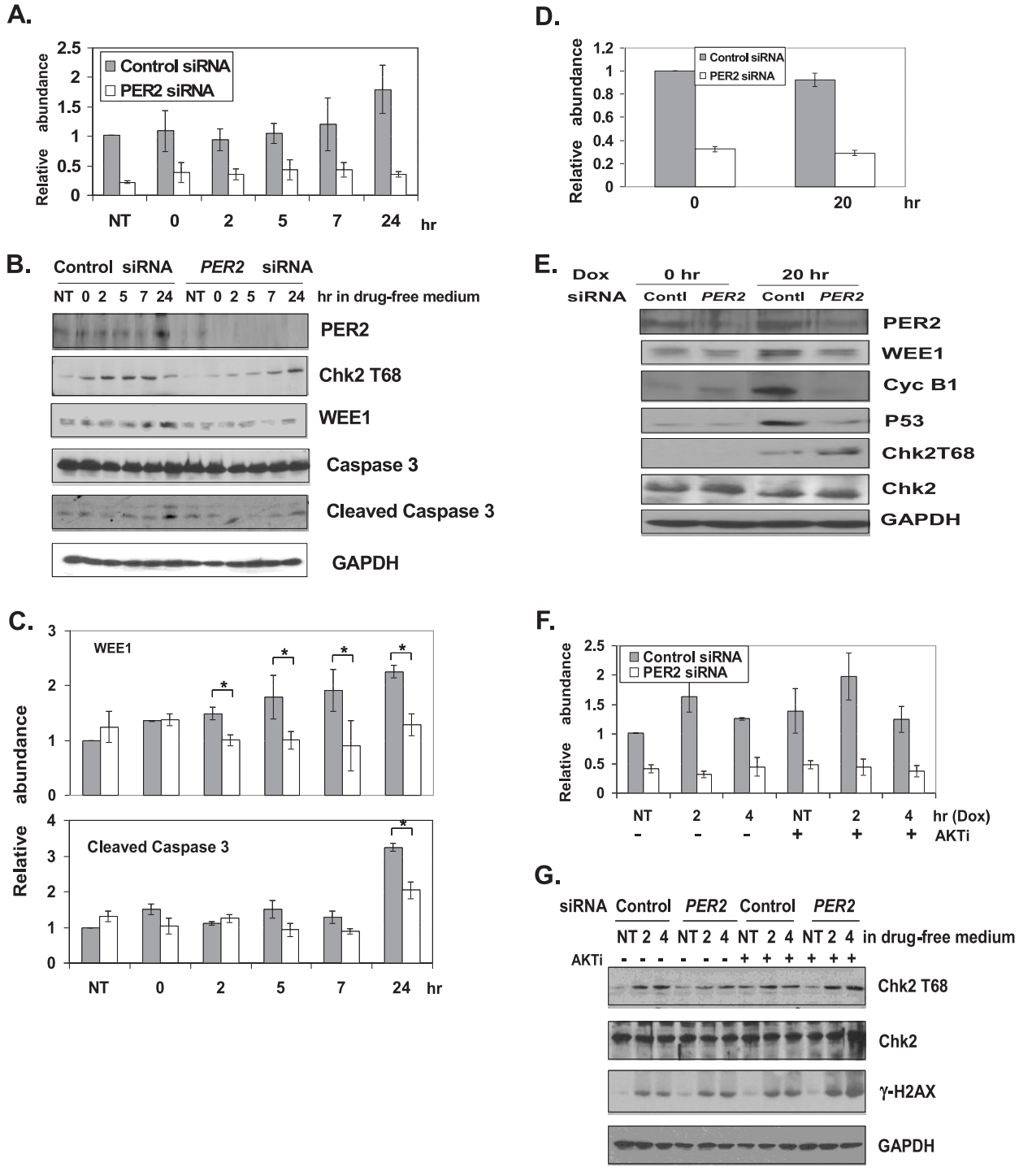
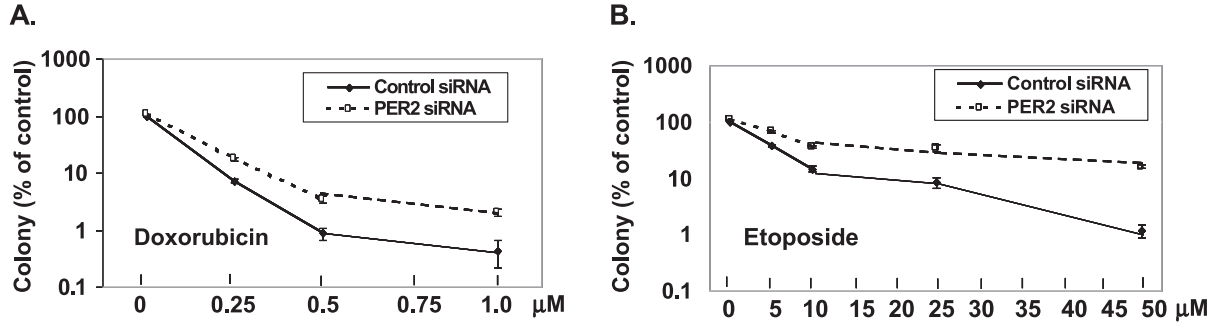


**Fig. 4.** Down-regulation of *PER2* enhances cancer cell resistance to chemotherapy drugs. After 48 h of siRNA treatment (Luciferase siRNA or *PER2* siRNA set 1), HCT116 cells were treated with the indicated concentrations of doxorubicin (A) or etoposide (B) for 24 h. Numbers of the colony were counted after 7 days of growth in the drug free medium. Colony number of the control knockdown cells without drug treatment was set as 100%.




Attenuated Chk2 activation in PER2-depleted cells can be prevented by inhibiting AKT activity, suggesting that PER2 modulates DNA damage response through AKT. It has been reported that down-regulation of *PER1* completely abolishes DNA damage-induced Chk2 T68 phosphorylation (Gery et al. 2006). A recent report shows that PER3, another homology of PER2, is also required for Chk2 activation and overexpression of PER3 leads to Chk2 activation without DNA damage (Im et al. 2010). However, the cancer prone phenotype has never been reported in *Per1* or *Per3* knockout mice. In *PER2* down-regulated cells, Chk2 T68 phosphorylation is attenuated and delayed but not completely abolished after DNA damage. It is unclear whether PER1, PER2, and PER3 exert their tumor suppressive functions through the same pathway. In mammalian circadian clockwork, PER1 and PER2 have similar but nonredundant functions, while PER3 seems not essential for the circadian clock (Bae et al. 2001; Zheng et al. 2001).

In summary, PER2 may exert its tumor suppressive function by regulating AKT activation. We will further determine how PER2 regulates AKT activation. PER2 is a transcription factor. It may also regulate cell proliferation and DNA damage response through other mechanisms. For example, PER2 could directly regulate the expression of genes such as Cyclin D, C-Myc, and p53. The transcription of these genes is deregulated in *Per2* mutant mice (Fu et al. 2002). It is unclear whether the deregulated expression of these genes is related to AKT over activation.

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