Letter to the Editor

Prohibitin 1 regulates tumor cell apoptosis via the interaction with X-linked inhibitor of apoptosis protein

Dear Editor,

Prohibitin 1 (PHB1) was identified previously as a protein that is upregulated and translocated to the plasma membrane in taxane-resistant cancer cells. We found that PHB1 silencing re-sensitized taxane-resistant cancer cells to apoptosis and to paclitaxel treatment both *in vitro* and *in vivo* (Patel et al., 2010). In line with our findings, overexpression of PHB1 has been shown to markedly attenuate ceramide-, staurosporine (STS)-, camptothecin-, or serum withdrawal-induced apoptosis via the intrinsic apoptotic pathway (Peng et al., 2015). In complementary studies, PHB1 silencing sensitized several cancer cell types to stress- or drug-induced apoptosis (Peng et al., 2015). Despite these advances, the mechanism whereby PHB1 regulates apoptosis and chemoresistance remains unclear. In this study, we identify X-linked inhibitor of apoptosis protein (XIAP) as a novel binding partner of PHB1 based on immunoprecipitation followed by mass spectrometry (IP-MS). We further show that this interaction is functionally relevant to the modulation of apoptotic process and tumor response to chemotherapeutic agents.

The identity of potential PHB1-interacting proteins was revealed by IP-MS-based proteomic analysis (Sowa et al., 2009). PHB1-HA is predominantly, but not solely, expressed in mitochondria, consistent with previous findings (Supplementary Figure S1A). The determined PHB1 interaction network (Supplementary Figure S1B and Table S1) includes several known PHB1-binding proteins, such as PHB2 and RAF1 (Peng et al., 2015), providing an internal validation of the approach. The screen also identified several novel PHB1-interacting proteins, including the serine β-lactamase-like

protein LACTB, the mitochondrial carbamoyl phosphate synthase 1 (CPS1), the mitochondrial ras family GTPases Rho T1 and T2, and the apoptosis inhibitor XIAP. These proteins participate in cellular functions including apoptosis, mitochondrial homeostasis, unfolded protein response, and signal transduction, where PHB1 plays important roles (Thuaud et al., 2013). The identification of XIAP, a well-characterized anti-apoptosis factor and potential cancer therapeutic target (Eckelman et al., 2006), as a PHB1 interactor offers the possibility that this interaction may mediate the known effects of PHB1 on apoptosis (Patel et al., 2010). Immunoprecipitation of HEK293 cells with anti-HA agarose validated the intracellular association of PHB1-HA with XIAP (Figure 1A). PHB2, a known PHB1 binding partner, was also found in the immunoprecipitates. More importantly, our results demonstrated that XIAP interacts with endogenous PHB1 in both Mes-Sa uterine sarcoma cells (Figure 1B) and OVCAR5 ovarian cancer cells (Supplementary Figure S1C). GST pull-down assays further revealed an interaction between $6 \times$ His-XIAP and GST-PHB1 *in vitro* (Figure 1C). Together, these results demonstrate that PHB1 binds directly to XIAP, both *in vivo* and *in vitro*.

XIAP contains three BIR domains. BIR1 directly interacts with TAB1 to induce NF-κB activation (Lu et al., 2007). BIR2 mediates binding of XIAP to downstream effector caspases (caspase-3 and caspase-7), whereas BIR3 binds to an upstream initiator caspase (caspase-9). BIR3 also mediates the binding to functional XIAP antagonists such as DIABLO, ARTS, and HtrA2/Omi (Eckelman et al., 2006). Co-immunoprecipitation (Co-IP) assays with His-V5-tagged PHB1 and multiple HA-tagged XIAP expression constructs,

including full-length XIAP, XIAPΔBIR, XIAP-BIR1-2, and XIAP-BIR2-3, revealed that PHB1 binds to full-length XIAP and the XIAP-BIR2-3 domain (Supplementary Figure S1D). Examination with HA-tagged PHB1 and more specific GST-tagged XIAP expression constructs, including full-length XIAP, XIAP-BIR1, XIAP-BIR2, and XIAP-BIR3, showed that PHB1 binds principally to the BIR3 domain of XIAP (Figure 1D) and weakly to the XIAP-BIR2 domain. We conclude that the principal PHB1-interacting site on XIAP resides within the BIR3 domain.

PHB1 is present in multiple compartments of the cell, principally in the mitochondria, but also in the cytosol, nucleus, and plasma membrane (Thuaud et al., 2013). To determine the regions where PHB1 and XIAP co-localize, we performed immunofluorescence double-labeling of cells with anti-PHB1 and anti-XIAP antibodies. In both Mes-Sa and OVCAR5 cells, XIAP localized primarily to the cytoplasm, and PHB1 was detected predominantly in the mitochondria, although some cytoplasmic staining was apparent (Supplementary Figure S2A). Moreover, when cells were treated with paclitaxel to induce apoptosis, cytoplasmic PHB1 staining increased and showed co-localization with cytoplasmic XIAP (Figure 1E and Supplementary Figure S2B). Subcellular fractionation followed by western blotting showed that, after treatment with paclitaxel, PHB1 levels were significantly elevated, in both mitochondria and cytosolic fractions (Supplementary Figure S2C). Additionally, we used the Duolink (Sigma) *in situ* proximity ligation assay to measure the intracellular PHB1–XIAP interaction. Immunofluorescence staining confirmed the silencing efficiency of both PHB1 and XIAP siRNAs and, importantly, the
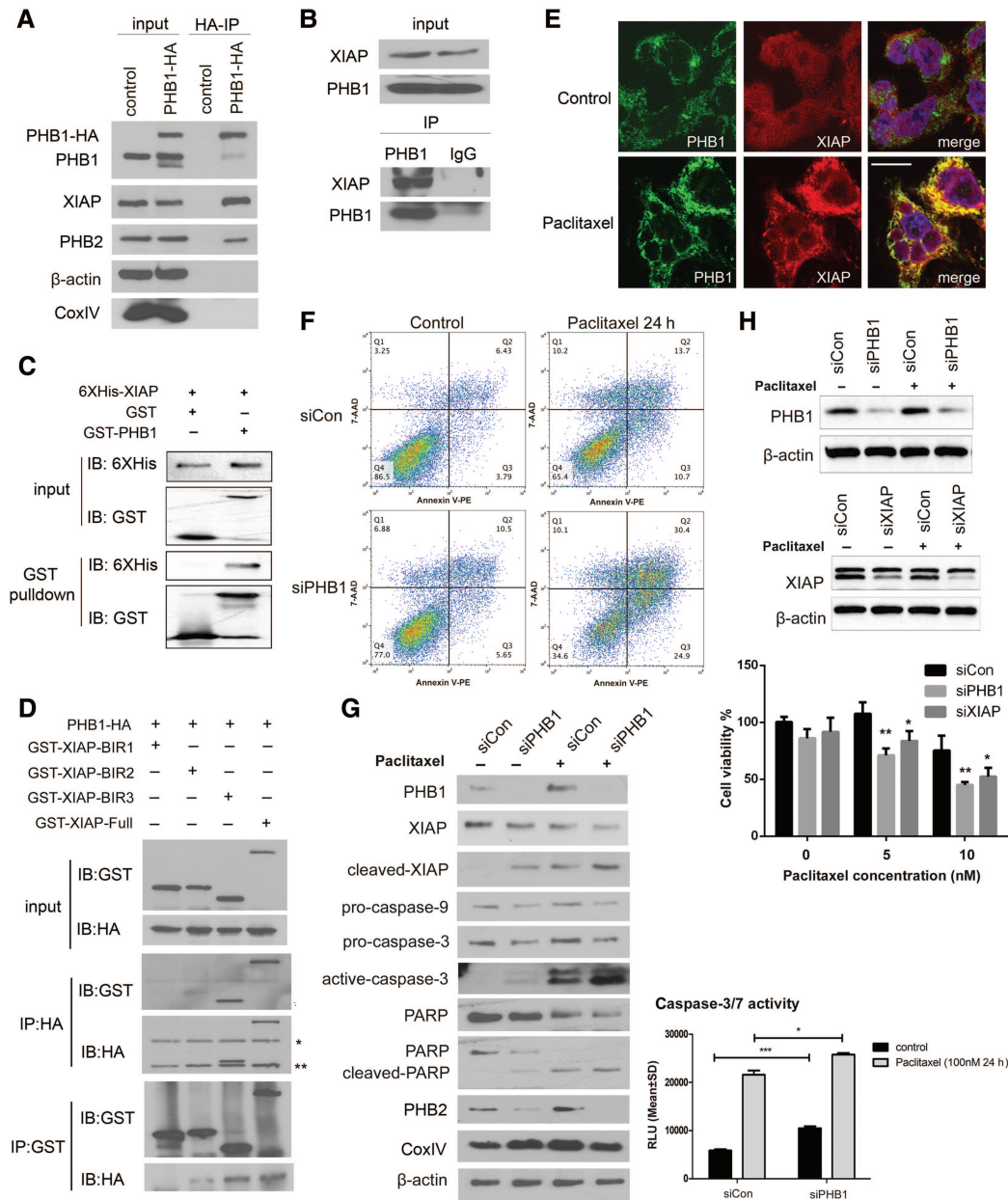
Figure 1 PHB1 binds to XIAP and regulates cell apoptosis. (**A**) Lysates of HEK293 transfected with PHB1-HA were subjected to HA agarose immunoprecipitation (IP) followed by western blotting. Input: 5% lysates related to the total IP. (**B**) Co-IP of endogenous PHB1 and XIAP in Mes-Sa cells. Lysates of Mes-Sa were subjected to anti-PHB1 IP followed by western blotting. Input: 5% lysates related to the total IP. (**C**) *In vitro* GST pull-down. Direct binding of PHB1 to XIAP is shown in an *in vitro* assay using GST-PHB1 and purified recombinant His-XIAP protein. GST protein served as a negative control. (**D**) HEK293 cells were transiently transfected with PHB1-HA along with GST-tagged full-length XIAP (GST-XIAP-Full) and XIAP mutation constructs (GST-XIAP-BIR1, GST-XIAP-BIR2, and GST-XIAP-BIR3). IP with anti-HA agarose or anti-GST antibody with agarose, followed by western blotting with anti-HA and anti-GST antibodies. Whole-cell lysates (input) showed that all constructs were expressed in the transfected cells. * indicates the heavy chain; ** indicates PHB1-HA. (**E**) Immunofluorescence of Mes-Sa cells stained with anti-PHB1 (green) and anti-XIAP (red) antibodies. The nucleus is stained with DAPI (blue). Upper: untreated cells; lower: cells treated with 500 nM paclitaxel for 16 h. Scale bar, 20 μm. (**F**) Mes-Sa cells were transfected with siCon or siPHB1 for 2 days, followed by paclitaxel (250 nM) treatment for 24 h. Results of flow cytometry analysis for apoptosis induction are presented in quadrants. (**G**) Mes-Sa cells were transfected with siCon or siPHB1 for 2 days, followed by paclitaxel treatment for 24 h. Left panel: western blotting of Mes-Sa cell lysates after treatment with 250 nM paclitaxel for 24 h. Right panel: caspase-3/7 activity after treatment with 100 nM paclitaxel for 24 h. Background activity values were subtracted from each sample. *$P < 0.05$; ***$P < 0.001$. Error bars are SDs. (**H**) Mes-Sa cells were transfected with siCon, siPHB1, or siXIAP for 2 days, followed by paclitaxel treatment for 24 h. Upper panel: western blotting for the efficiency of PHB1 and XIAP knockdown. Lower panel: cell viability determined by CyQUANT cell viability assay in three independent experiments. *$P < 0.05$; **$P < 0.01$. Error bars are SDs.

specificity of antibodies used (Supplementary Figure S3A). The experiment (Supplementary Figure S3B) revealed a small amount of Duolink signal (red fluorescent puncta) under control conditions along with a clear increase in the number of cytoplasmic interaction events after paclitaxel treatment. Together, these results suggest that the XIAP–PHB1 interaction takes place predominantly in the cytoplasm and is increased following paclitaxel-induced apoptosis.

To confirm the involvement of PHB1 in apoptosis in these cells, we silenced PHB1 in Mes-Sa cells using PHB1-specific siRNA followed by paclitaxel treatment to induce apoptosis. By flow cytometry analysis for Annexin-V (apoptosis marker) and 7-AAD (necrosis marker), we found that PHB1 silencing sensitized cells to paclitaxel-induced apoptosis (Figure 1F). After paclitaxel treatment, the frequency of apoptosis, as demonstrated by the percentage of Annexin-V-positive cells, increased markedly from 24.4% in siCon cells to 55.3% in siPHB1 cells. Early-stage apoptosis, marked by the percentage of Annexin V-positive/7-ADD-negative cells, similarly increased from 10.7% in siControl cells to 24.9% in siPHB1 cells. In line with this finding, early-stage apoptosis was increased in Mes-Sa cells after treatment with the well-characterized apoptosis inducer STS (Supplementary Figure S4A). Although the time frames for apoptosis caused by paclitaxel and STS are different, PHB1 silencing increased apoptosis in both cases, whereas non-apoptotic cell death (7AAD-positive only) remained relatively constant.

One possible mechanism could be that PHB1 competes with other BIR domain-binding proteins, such as the well-known XIAP antagonist DIABLO, to bind to the XIAP-BIR3 domain. If this were the case, PHB1 silencing should result in an increased level of the XIAP–DIABLO complex. Our results, however, showed decreased XIAP–DIABLO interaction, as well as the interaction of XIAP with caspase-9, another BIR3 domain-binding protein, after PHB1 silencing in the presence of paclitaxel or STS (Supplementary Figure S5A and B). Thus, the results do not support a direct competition for XIAP binding between prohibitin and DIABLO or caspase-9. But the levels of full-length XIAP were partly decreased after PHB1 silencing, suggesting a potential effect of PHB1 on XIAP stability.

It was shown previously that XIAP is cleaved by caspases at aspartic acid-242 between the BIR2 and BIR3 domains. The resulting BIR1-2 cleavage product is degraded, while the BIR3-RING domain product acts as a part of a positive feedback loop to increase apoptosis (Hornle et al., 2011). We speculated that PHB1 binding to the XIAP-BIR3 domain could interfere with caspase-mediated XIAP cleavage. siCon and siPHB1 Mes-Sa cells were treated with paclitaxel to induce apoptosis, and the levels of XIAP and its downstream effectors were measured. Western blotting showed that PHB1 silencing leads to the generation of a 30-kDa cleaved-XIAP BIR3-RING domain, which resulted in enhanced caspase-3 processing into the catalytically active p17 fragment (cleaved caspase-3) and a consequent increase in cleaved-PARP, which was augmented by paclitaxel (Figure 1G, left panel). The XIAP staining observed in paclitaxel-treated cells in Figure 1E likely represents both full-length and cleaved XIAP, recognized by antibody to the XIAP C-terminus. Moreover, Caspase-Glo assay revealed that PHB1 silencing leads to increased caspase-3/7 activity following paclitaxel treatment (Figure 1G, right panel). These findings are consistent with a role for PHB1 as an inhibitor of apoptosis and were verified further in multiple cell lines (Supplementary Figure S4B and C). Thus, the functional phenotypes of PHB1–XIAP are not cell type-specific. Our study shows that PHB1 protects XIAP functionality by diminishing caspase-mediated XIAP cleavage. PHB1 was previously described as a chaperone for mitochondrial proteins. Our results suggest a similar role for PHB1 in protecting cytoplasmic XIAP.

We next investigated whether PHB1 and XIAP have similar functionality. PHB1 or XIAP was silenced in Mes-Sa cells, followed with paclitaxel treatment. Figure 1H shows that silencing either PHB1 or XIAP increased cell killing by paclitaxel. Furthermore, we found that overexpression of XIAP in PHB1-silenced cells partially rescued the enhanced cell death caused by siPHB1 (Supplementary Figure S6A). PHB1 silencing significantly reduced the protein level of His-V5-XIAP (Supplementary Figure S6A), which was restored by treatment with the proteasome inhibitor MG132 (Supplementary Figure S6B). Together, these results further support that XIAP is protected from proteasomal degradation in the presence of PHB1.

The observation that PHB1 silencing increases cell death has potential clinical implications. PHB1 levels correlate with disease progression and chemoresistance in several cancer types, including gastric cancer, colorectal cancer, hepatocellular carcinomas, non-small cell lung cancer (NSCLC), and ovarian cancer (Kapoor, 2013). Interestingly, Gregory-Bass et al. (2008) showed a strong positive correlation between the levels of PHB1 and XIAP in ovarian cancer, suggesting that PHB1 may be coordinately regulated and exert an anti-apoptotic effect in ovarian cancer cells. Moreover, we observed that PHB1 silencing leads to the loss of PHB2 expression (Figure 1G, Supplementary Figure S4B and C). These findings provide further evidence for the interdependence between PHB1 and PHB2, which have a propensity to form stable oligomers (Thuaud et al., 2013; Peng et al., 2015), suggesting that cellular defects observed in PHB1-silenced cells may be attributable to the loss of the prohibitin complex. The newly identified PHB1 interactome may pave the way for a deeper understanding of the role for the prohibitin complex in regulating mitochondrial activity, cell survival, and apoptosis.

In summary, we have demonstrated an interaction between PHB1 and XIAP and suggest a novel mechanism for the suppression of apoptosis by PHB1. Our findings indicate that the PHB1–XIAP complex promotes an anti-apoptotic response in cancer cells and reinforces PHB1 as a therapeutic target, as PHB1 silencing increases the sensitivity of cancer cells to drug-induced apoptosis. The feasibility of employing PHB1 as a therapeutic target was further demonstrated by our recent finding that systemic delivery of PHB1-siRNA nanoparticles could increase the susceptibility to cisplatin in NSCLC xenografts in mice (Zhu et al., 2015).

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