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Prohibitin Ligands in Cell Death and Survival: Mode of Action and Therapeutic Potential

Frédéric Thuaud, Nigel Ribeiro, Canan G. Nebigil, and Laurent Désaubry

Introduction
Prohibitin-1 (PHB1, formerly known as BAP32) and its homolog PHB2 (formerly BAP37, REA, or prohibitone) are pleiotropic proteins with multiple functions. PHB1 was initially identified in 1989 by McClung and collaborators by screening for potential tumor suppressors with antiproliferative activities that were highly expressed in normal resting, but not regenerating, rat liver (McClung et al., 1989; Nuell et al., 1991). PHBs have since been shown to act as a hub for many signaling pathways triggered by growth factors, the immune response, and steroid hormones regulating metabolism, mitochondrial biogenesis, cell migration, division, and survival.

PHBs are composed of an N-terminal transmembrane domain, an evolutionarily conserved PHB domain that is common to other scaffold proteins (including stomatin, flotillin, and Hflk/C), and a C-terminal coiled-coil domain that is involved in the interaction between PHB1 and PHB2 (Figure S1 available online). Although the structure of PHBs has not been solved, Winter et al. (2007) were able to generate structural models of human PHBs based on the known structure of flotillin-2, which belongs to the same family of membrane proteins.

Mitochondrial Functions of PHBs
Located in the mitochondrial inner membrane, PHB1 and PHB2 interact with each other to form heterodimers that are organized in ring-like structures with a diameter of 20–25 nm (Figure 1; Back et al., 2002; Coates et al., 2001). These supercomplexes maintain the structure of mitochondria and regulate their functions (Tatsuta et al., 2005). PHBs protect newly imported proteins from degradation by the m-AAA (mitochondrial ATPases Associated with diverse cellular Activities) protease (Steglich et al., 1999), promote mitochondrial protein synthesis (He et al., 2012), maintain the organization and copy number of mitochondrial DNA (mtDNA; Kasashima et al., 2008), and act as a chaperone for newly synthesized proteins of the mitochondrial complex I (Nijtmans et al., 2000, 2002) and the GTPase optic atrophy 1 (Opa1) during mitochondrial fission and morphogenesis (Merkwirth et al., 2008). PHB1 also interacts with the adaptor protein p66Shc, a major mediator of stress-induced apoptosis (Madireddi, 2006). This adaptor protein is activated by UVC, H2O2, and reactive oxygen species (ROS), and induces the generation of ROS in mitochondria, leading to apoptosis (Gertz et al., 2008). It remains unclear whether PHB1 modulates this proapoptotic response; nevertheless, it is tempting to speculate that p66Shc is involved in the cytoprotective activities of PHBs and their ligands, particularly against oxidative stress (Bernard et al., 2011; Fahrig et al., 2005; Kathiria et al., 2012a; Liu et al., 2009b; Ribeiro et al., 2012a).

PHB2 is also regulated by a second messenger, sphingosine-1-phosphate (S1P), which controls diverse physiological processes through several targets. S1P binds with high affinity to PHB2 and thereby maintains the integrity of the mitochondrial respiration machinery (Strub et al., 2011). This process is involved in the preconditioning protection of the heart against ischemia-reperfusion injury (Gomez et al., 2011).

Regulation and Physiological Role of PHBs
PHBs are not limited to mitochondria; they are also found in the nucleus, the endoplasmic reticulum, the plasma membrane, and macrophage phagosomes (Garin et al., 2001), where they modulate many aspects of cell physiology. Their roles are extremely complex, partly because PHBs are themselves regulated by several tyrosine and serine phosphorylations, O-GlcNAc modifications, palmitoylations, transamidations, and tyrosine nitrosylations (Mishra et al., 2010). PHB1 interacts physically with the second messenger PIP3, and PHB2 interacts with S1P (Ande and Mishra, 2009; Strub et al., 2011).

Each posttranslational modification of the PHBs has substantial effects on their activity (Figure 2). One of the best-documented examples is Akt phosphorylation of PHB1 at Thr258, which blocks its interaction with Shp1/2 and facilitates Akt signaling (Ande and Mishra, 2009). Shp1/2 is a phosphatase that facilitates Akt signaling and enhances insulin signaling. Additionally, the phosphorylation of PHB1 at Thr258 is necessary for the activation of C-Raf (Raf-1) by Ras (Chiu et al., 2013). Rajalingam et al. (2005) established that the activation of C-Raf by Ras requires the heterodimerization of phospho-PHB1(Thr258) with C-Raf. The phosphorylation of PHB1 at
Thr258 in the plasma membrane of cancer cells activates PI3K/Akt and C-Raf/ERK pathways, which promote proliferation and metastasis (Chiu et al., 2012). By contrast, insulin-receptor-induced phosphorylation of PHB1 at Tyr114 promotes its heterodimerization with the phosphatase Shp1 and blocks Akt signaling (Ande et al., 2009a).

It has been suggested that the regulation of PHB1 by transforming growth factor β (TGF-β) is responsible for the dual effect of this cytokine in prostate cancer (Zhu et al., 2010). In the early stage of tumorigenesis, TGF-β acts as a tumor suppressor, but subsequently it promotes metastatic spread during cancer progression. The binding of TGF-β to its receptor triggers the C-Raf/MEK/ERK pathway and also activates Smad 2/3 or Smad 1/5, thereby causing opposite effects (Figure 2). ERK activates protein kinase C (PKC), leading to the phosphorylation of PHB1 and consequently to cell survival and invasion. However, Smad signaling also upregulates 14-3-3 protein, which inhibits PKC-δ, leading to a hypophosphorylation of PHB1 that promotes apoptosis.

The activity of PHB2 is regulated by serine and threonine phosphorylation, in particular at Ser91 and Tyr248 (Ross et al., 2008; Sun et al., 2011). Calcium/calmodulin-dependent protein kinase IV (CaMKIV) phosphorylates PHB2 at Ser91, impeding its ability to repress the transcriptional activity of myocyte enhancer factor 2 (MEF2; Sun et al., 2011). The consequence of this phosphorylation of PHB2 on the activity of other transcription factors has not been reported. PHB2 also inhibits circadian transcription by interacting with casein kinase 1 ε (CK1ε; Kategaya et al., 2012).

PHBs have been detected at the surface of platelets, microglial cells (Wintachai et al., 2012), endothelial cells in adipose tissue (Kolonin et al., 2004), paclitaxel-resistant cancer cells (Patel et al., 2010), intestinal epithelial cells (Sharma and Qadri, 2004), and activated T cells (Yurugi et al., 2012). In platelets, PHB1 and PHB2 interact directly with protease-activated receptor 1 (PAR1) to promote platelet aggregation (Zhang et al., 2012). The physiological role of PHB1 at the surface of microglial cells and endothelial cells of adipose tissue remains elusive, but it has been demonstrated that PHB1 at the surface of cancer cells is involved in resistance to paclitaxel (Patel et al., 2010). A virulence antigen of Salmonella typhi, interacts with PHB1 and PHB2 at the surface of intestinal epithelial cells, resulting in downregulation of the mitogen-activated protein kinase (MAPK) cascade and inhibition of early inflammatory responses (Patel et al., 2010). Similarly, considerable proportions of total PHB1 and PHB2 are located on the surface of activated T cells, where they contribute to the activation of ERK induced by T cell activation (Yurugi et al., 2012).

PHB1 is also present in human serum, embedded in lipid droplets where it interacts with the complement protein C3 to promote innate immunity (Mishra et al., 2007). Interestingly, serum PHB1 and PHB2 concentrations were found to be significantly elevated in patients with colorectal cancer, suggesting that they may serve as a biomarker for this type of cancer (Mengwasser et al., 2004).

A brief presentation of the interactions between PHBs and other proteins is provided in Table 1 (for a list of other putative partners of PHB1, see Table S1).

**PHBs and Cancer**

Both PHB1 and PHB2 are involved in growth, resistance to chemotherapy, and metastasis through several mechanisms, including activation of the Ras-C-Raf-MEK-ERK pathway, modulation of TGF-β signaling, and transcriptional regulation. Indeed, PHB1 interacts with the transcription factor p53 in the nucleus of cancer cells to increase its transcriptional activity (Fusaro et al., 2003). p53 is a tumor suppressor that controls the cell cycle, apoptosis, genomic stability, and angiogenesis (Chander et al., 2010, 2011; Fusaro et al., 2003; Joshi et al., 2007). A component of ubiquitin ligase complexes, Skp2B, which is overexpressed in many breast cancers, interacts with both PHB1 and PHB2 to promote their degradation (Chander et al., 2010, 2011; Umanskaya et al., 2007). Because PHB1 acts as an activator (Fusaro et al., 2003) and chaperone (Chander et al., 2011) for p53, this depletion in PHB1 leads to an attenuated activity of p53 in cancer cells overexpressing Skp2B and consequently promotes their survival.

PHB1 is also an important regulator of the retinoblastoma tumor suppressor protein (Rb) and its family members P107 and p130 (Wang et al., 1999b). These proteins bind and inhibit
E2F transcription factors, leading to cell-cycle arrest. PHB1 represses the activity of E2F transcription factors by interacting with several proteins, including Rb, histone deacetylases (HDACs), and the nucleosome-remodeling proteins Brg-1 and Brm (Wang et al., 1999a, 1999b, 2002).

A growing body of evidence indicates that the cellular localization of PHBs is a determinant of their function, especially in cancer cells. Patel et al. (2010) found that PHB1 is overexpressed on the surface of cancer cells that are resistant to taxoids, doxorubicin, and etoposide. They also demonstrated that the redirection of PHB1 from the cytoplasm to the cell surface is critical for drug resistance, and their work suggests that cell-surface PHB1 may serve as a biomarker of chemoresistance in cancer patients.

In normal epithelial breast cells, PHB1 is located primarily in the mitochondria, but in invasive and noninvasive breast cancer cells it is mainly confined to the nucleus, suggesting that PHB1 localization is associated with tumorigenesis (Chen et al., 2011). Confocal microscopy experiments demonstrated that PHBs colocalize with the proto-oncogenes c-myc, c-fos, p53, and Rb in neuroblasticoma SK-N-SH cells (Li et al., 2011).

Treatment with the anticancer drug camptothecin leads to PHB1 and p53 being exported from the nucleus to the mitochondria of breast cancer cell lines (Fusaro et al., 2003). This camptothecin-induced translocation of PHB1, which is dependent on the protein transporter CRM-1, occurs preferentially in transformed cell lines and not in untransformed or primary cells. A peptide corresponding to the nuclear export signal of PHB1 prevented the export of PHB1, resulting in the protection of cells from apoptosis, indicating that the translocation of PHB1 from the nucleus to the mitochondria contributes to its tumor-suppressive effects (Rastogi et al., 2006b). One mechanism by which the intracellular location of PHBs is controlled involves the signaling protein BIG3/WDR5, which traps PHB2 in the cytoplasm and consequently stimulates the estrogen-signaling pathway in the hormone-related growth of breast cancer cells (Kim et al., 2009).

Beyond these activities of PHBs as proteins, the 3'-UTR of PHB1 messenger RNA (mRNA) was found to suppress both the proliferation of human breast cancer cells in vitro and the growth of breast tumors in xenografted mice in vivo (Manjeshwar et al., 2003). This was the first demonstration that an RNA molecule can function as a tumor suppressor in human breast cancer.

The tumor-suppressor activity of PHB1 and its mRNA 3'-UTR is further supported by the discovery that the oncogenic microRNA-27a (miR-27a), which is upregulated in many cancers (Chhabra et al., 2010), targets the 3'-UTR of PHB1 and downregulates PHB1 in human gastric adenocarcinoma (Liu et al., 2009a). Fletcher et al. (2012) demonstrated that miR-27a is upregulated by androgen receptor in prostate cancer, resulting in reduced PHB1 mRNA and protein levels and increased cancer cell growth. Interestingly, adding PHB1 to cells overexpressing miR-27a blocked miR-27a-induced growth, demonstrating that the oncogenic activity of miR-27a strongly involves PHB1 signaling in prostate cancer.
An important question that needs to be examined is why PHBs display both anti- and protumorigenic roles in cancers (summarized in Table 2). This dichotomy may reflect the heterogeneity of cancer cell types, which are manifested by an alteration of different signaling pathways. Consequently, the regulation of transcription, translation, and posttranslational modification of PHBs probably depends on the cancerous cell type involved. These posttranslational events (e.g., phosphorylation, SUMOylation, O-GlcNAc modification, palmitoylation, and transamidation) modulate both the subcellular localization of PHBs and their downstream effects on proliferation and survival of cells. On the cell surface PHB1 promotes chemoresistance, but on the inner face of the plasma membrane it can contribute to the oncogenic activation of C-Raf. However, it is tempting to speculate that in the nucleus PHB1 displays antitumorigenic activity due to its action on p53, Rb, p107, p130, and components of the mammalian replication machinery. Additional studies are required to decipher the roles played by signaling and intracellular localization of PHBs in oncogenesis.

**PHBs and Cytoprotection**

Many studies have indicated that PHBs protect cells from oxidative stress, which is due to an excessive production or an impaired elimination of ROS (Jones, 2008). It is closely associated with mitochondrial dysfunction and is an important component of the etiology of cancers, inflammatory, cardiovascular, and neurodegenerative diseases, and diabetes mellitus. In view of the central role of PHBs in maintaining the normal structure and function of mitochondria, it is unsurprising that PHBs can alleviate the deleterious effect of oxidative stress.

Many stresses, in particular oxidative stress, upregulate PHB1 and PHB2 to promote cell survival. Indeed, PHB1 expression is enhanced in neurons by various stresses, including electrical stimulation, hypoxia-ischemia, oxygen-glucose deprivation (Zhou et al., 2012), exercise-induced neuroplasticity (Ding et al., 2006), injection of the neurotoxin 6-hydroxydopamine (Park et al., 2010), and schizophrenia-induced oligodendrocyte dysfunction (Bernstein et al., 2012). PHB1 is also upregulated in the liver of steatohepatitis patients (Tsutsumi et al., 2009), in fetal rabbit lung after exposure to hyperoxic conditions (Henschke et al., 2006), in pancreatic β-cells after ethanol intoxication, (Lee et al., 2010b), and in cardiac cells after ischemic-hypoxic preconditioning (Kim et al., 2006; Muraguchi et al., 2010) or chronic restraint stress (Liu et al., 2004). This cytoprotective response involves the translocation of PHB1 from the nucleus and the cytoplasm to mitochondria in pancreatic β-cells (Lee et al., 2010b), in the retina and retinal epithelium (Lee et al., 2010a), and in ovarian granulosa cells (Chowdhury et al., 2007; Figure S2). Sripathi et al. (2011) suggested that the localization and trafficking of PHBs are determined by the modulation of their binding to specific ligands. Indeed, strong binding of PHB1 to cardiolipin was shown to correlate with the localization of PHB1 within mitochondria in transformed epithelial cells after an oxidative stress. By contrast, under normal conditions in these cells, PHB1 binds strongly to PIP3 but not to cardiolipin.

Overexpression of PHB1 protects pancreatic β-cells, ovarian granulosa cells, and cardiomyocytes from apoptosis induced by ethanol, ceramide, staurosporine, serum withdrawal, and oxidative stress-induced injury, consistent with PHB1 having a cytoprotective role (Chowdhury et al., 2007, 2011; Lee et al., 2010b; Liu et al., 2009b). Merkwirth et al. (2012) recently described a critical role of PHB2 in the survival of neurons. Neuron-specific deletion of PHB2 in the mouse forebrain impaired mitochondrial architecture, leading to tau hyperphosphorylation and filament formation. This phenotype was accompanied by severe behavioral and cognitive dysfunctions that were reminiscent of Alzheimer’s disease.

The details of the mechanisms of this cytoprotection remain poorly documented. The only other actors that have been identified as being involved are S1P and STAT3. S1P protects the heart from ischemia-reperfusion damage by directly activating PHB2, thereby regulating respiration and cytochrome oxidase subunit IV assembly and decreasing the heart’s susceptibility to opening of the permeability transition pore (PTP; Gomez et al., 2011). Interleukin-6 (IL-6) increases PHB1 levels through phosphorylation of the transcription factor STAT3 to promote the survival of intestinal cells and cardiomyocytes (Gratia et al., 2012; Theiss et al., 2007b). In intestinal epithelial cells in vivo, PHB1 interacts with STAT3 to modulate STAT3-mediated apoptosis (Kathiria et al., 2012b). In cardiomyocytes, IL-6-induced upregulation of PHB1 is central to the cardioprotective effects of IL-6 against oxidative stress (Gratia et al., 2012). Importantly, phosphorylated STAT3 protects cardiomyocytes against oxidative stress by stimulating respiration and inhibiting the PTP within mitochondria, and not through a transcriptional effect (Boengler et al., 2010). It would be interesting to determine whether PHB1 modulates this mitochondrial action of STAT3.

**PHBs and Inflammatory Diseases**

Inflammatory bowel diseases (IBDs) are characterized by strong oxidative stresses associated with downregulated antioxidant enzymes in the intestinal mucosa (Lih-Brody et al., 1996). Several studies in vitro and in vivo have indicated that PHB1 alleviates intestinal inflammation and promotes the survival of cells exposed to oxidative stress. In intestinal epithelial cells, PHB1 binds to a Salmonella typhi antigen to inhibit the inflammatory response to S. typhi infection (Sharma and Qadri, 2004). PHB1 expression is abnormally low during ulcerative colitis and Crohn’s disease, the two most common forms of IBD (Hsieh et al., 2006; Theiss et al., 2007a; Yeo et al., 2006), due to an overstimulation of tumor necrosis factor α (TNF-α) signaling in the inflamed colon (Theiss et al., 2009a). This downregulation of PHB1 increases ROS signaling and TNF-α-induced autophagy, promoting inflammation in patients with IBD (Kathiria et al., 2012a). Interestingly, transgenic mice overexpressing PHB1 in intestinal epithelial cells exhibit reduced proinflammatory nuclear factor κB (NF-κB) signaling in the colonic mucosa after treatment with TNF-α, demonstrating that PHB1 plays a critical role in inflammation. A detailed examination of this mechanism of action revealed that PHB1 inhibits TNF-α-induced translocation of NF-κB by downregulating the expression of importin α3, a protein involved in NF-κB nuclear import (Theiss et al., 2009a). In an in vivo model of IBD, PHB1 transgenic mice exhibited lower oxidative stress and colitis than wild-type mice (Theiss et al., 2009b). This cytoprotective effect is due to the upregulation of nuclear factor erythroid 2-related factor 2 (Nrf2), a transcriptional activator of...
| Binding Partners | PHB  | Biological Consequences                                                                 | References                |
|------------------|------|----------------------------------------------------------------------------------------|---------------------------|
| **Mitochondrial Proteins** |      |                                                                                         |                           |
| m-AAA protease   | PHB1 | regulation of membrane protein degradation                                               | Steglich et al., 1999     |
| ATAD3            | PHB1 | mitochondrial protein synthesis                                                          | He et al., 2012           |
| nucleoids (mitochondrial DNA associated to nucleoproteins)  | PHB1/2 | maintenance of the organization and copy number of the mtDNA                          | Kasashima et al., 2008   |
| dynamin-like GTPase OPA-1 | PHB1  | stabilization of the structure of mitochondria during their fusion and morphogenesis   | Merkwirth et al., 2008   |
| subunits of cytochrome c oxidase | PHB1  | function of the mitochondrial respiratory chain                                          | Tsutsumi et al., 2009    |
| NADH-ubiquinone oxidoreductase 30 kDa subunit | PHB1  | stabilization of mitochondrial complex I                                                | Park et al., 2010         |
| ND4 and ND5      | PHB1/2 | assembly of mitochondrial complex I                                                     | Bourges et al., 2004      |
| SLP-2            | PHB1  | stabilization of PHBs                                                                   | Da et al., 2008           |
| TFAM             | PHB1  | regulation of copy number of mitochondrial DNA                                          | Kasashima et al., 2008   |
| ANT2             | PHB2  | unknown                                                                                 | Kasashima et al., 2006   |
| VDAC2            | PHB2  | unknown                                                                                 | Kasashima et al., 2006   |
| Hax-1            | PHB2  | stabilization of antiapoptotic Hax-1                                                   | Kasashima et al., 2006   |
| nitric oxide-associated protein 1 (mNOA1) | PHB1  | regulation of mitochondrial protein translation and respiration                         | Heidler et al., 2011      |
| phosphorylated p66ShcA | PHB1  | putative protection against oxidative stress                                            | Madireddi, 2006           |
| **Transcription Factors** |      |                                                                                         |                           |
| estrogen receptor | PHB1/2 | repression of estrogen-receptor activity                                                  | Delage-Mourroux et al., 2000; He et al., 2008; Montano et al., 1999 |
| E2Fs             | PHB1  | repression of E2F activities                                                             | Gamble et al., 2007; Joshi et al., 2003, 2007; Schneider et al., 2010 |
| p53              | PHB1  | enhancement of p53-mediated transcription activity and chaperone activity of PHB1 for p53 | Chander et al., 2011; Fusaro et al., 2003; Joshi et al., 2007 |
| MyoD and MEF2    | PHB2  | repression of MyoD and MEF2 activities                                                   | Sun et al., 2004, 2011    |
| chicken ovalbumin upstream binding transcription factors I and II | PHB2  | transcriptional repression                                                               | Kurttev et al., 2004      |
| phosphorylated STAT3 | PHB1  | modulation of p53- and STAT3-mediated apoptosis                                           | Kathiria et al., 2012b    |
| **Other Nuclear Proteins** |      |                                                                                         |                           |
| HDAC1 and HDAC5  | PHB1/2 | transcripional repression                                                                | Kurttev et al., 2004      |
| Rb and its family members p107 and p130 | PHB1  | repression of E2F-mediated transcription and inhibition of cell proliferation            | Wang et al., 1999a, 1999b, 2002 |
| Brg-1 and Brm    | PHB1  | repression of E2F-mediated transcription and inhibition of cell proliferation            | Wang et al., 2002         |
| heterochromatin protein 1 (HP1) family proteins | PHB1  | repression of E2F-mediated transcription and induction of cellular senescence           | Rastogi et al., 2006a     |
| RING finger protein 2 (RNF2) | PHB1/2 | repression of the transcriptional activity of E2F1 and CP2c                             | Choi et al., 2008; Lee et al., 2008 |
| histone methyltransferase EZH2 | PHB2  | repression of estrogen-dependent transcription                                           | Hwang et al., 2008        |
| PGC1α (PPARγ coactivator 1) | PHB2  | inhibition of the transcriptional activity of PPARγ                                     | Endo, 2011                |

(Continued on next page)
### Table 1. Continued

| Binding Partners | PHB | Biological Consequences | References |
|------------------|-----|-------------------------|------------|
| minichromosomes maintenance complex of proteins MCM2, MCM5, and MCM 7 | PHB1 | inhibition of DNA replication | Rizwani et al., 2009 |
| nuclear receptor corepressor 1 (NCoR) | PHB1 | transcriptional repression of E2F family members | Wang et al., 2002 |

#### Other Signaling Proteins

| IgM receptor | PBH1 | phosphorylation of PHB1 | Terashima et al., 1994 |
| protease-activated receptor 1 | PHB1/2 | platelet aggregation | Zhang et al., 2012 |
| C-Raf | PHB1 | phosphorylation of C-Raf by Ras and consequently activation of the MEK-elf4 pathway | Chiu et al., 2012; Chiu et al., 2013; Rajalingam et al., 2005 |
| Akt2 | PHB2 | cell-cycle exit and myogenic differentiation | Héron-Milhavet et al., 2008; Sun et al., 2004 |
| WD repeat domain 5 (WDR5, BIG3) | PHB2 | inhibition of the translocation of PHB2 to the nucleus | Kim et al., 2009 |
| Skp2B | PHB1/2 | attenuation of the transcriptional activity of p53, release of the repression of the estrogen receptor | Chander et al., 2010, 2011; Umanskaya et al., 2007 |
| MLK2 (mixed lineage kinase 2) | PHB1 | unknown | Rasmussen et al., 1998 |
| Shp1 (phosphatase) | phospho-Y114-PHB1 | modulation of insulin signaling | Ande et al., 2009a |
| PKC-δ | PHB1 | phosphorylation of PHB1 leading to a decrease in the inner mitochondrial membrane permeability and to cell survival | Zhu et al., 2010 |
| 14-3-3 protein | PHB1 | inhibition of PHB1 phosphorylation leading to an increase in the inner mitochondrial membrane permeability and to apoptosis | Zhu et al., 2010 |
| α-dystrobrevin | PHB1 | NB4 cell granulocytic differentiation | Borutinskaite et al., 2011 |
| transient Receptor Potential Melastatin 6 (TRPM6) | PHB2 | inhibition of TRPM6-mediated transepithelial Mg²⁺ transport | Cao et al., 2009 |
| HSP70 | PHB1 | resistance to stress | Liu et al., 2010 |
| glucose-regulated protein 75 (GRP75)/mortalin | PHB1 | cell proliferation, tumorigenesis and stress response in metastatic cells | Martin et al., 2008 |
| phospho- Ser²⁹¹- Syk | PHB1 | B cell antigen-receptor signaling | Paris et al., 2010 |
| CRM-1 | PHB1 | export of PHB1 outside of the nucleus selectively in cancer cells | Rastogi et al., 2006b |
| Pex14p | PHB1 | regulation of the peroxisomal importomer | Oeljeklaus et al., 2012 |
| annexin A2 | PHB1/2 | interaction blocked by calcium, association with lipid rafts | Zhu et al., 2010 |
| alpha-actinin | PHB1/2 | interaction blocked by calcium, association with lipid rafts | Bacher et al., 2002 |
| EHD2 | Palmitoylated PHB1 | inhibition of pyruvate carboxylase leading to a decrease in glucose and fatty acid oxidation | Ande and Mishra, 2010; Vessal et al., 2006 |
| C3 | PHB1 | enhancement of innate immunity response | Mishra et al., 2007 |
| CK1ε | PHB2 | inhibition of circadian transcription | Kategaya et al., 2012 |

#### Proteins of Pathogens

| SV40Tag (viral oncoprotein) | PHB1 | interruption of the association between PHB1 and Brg-1/Brm | Wang et al., 2002 |
| HIV-1 glycoprotein | PHB1/2 | viral spread in nonpermissive cells | Emerson et al., 2010 |
| DENV-2 E protein | PHB2 | internalization of the dengue virus | Kuadkitikan et al., 2010 |
antioxidant responses. Further evidence that PHB1 is a putative therapeutic target for treating IBD comes from the observations that adenovirus-directed administration by enema and nanoparticle-based colonic delivery of PHB1 effectively enhanced the levels of PHB1 at the surface of colonic epithelial cells and alleviated colitis induced by dextran sodium sulfate (Theiss et al., 2011).

Picard et al. (2013) recently demonstrated that PHB1 accumulates in the nucleus of osteoarthritic chondrocytes, where it represses the expression of the transcription factor PITX1, suggesting an involvement of PHB1 in the etiology of osteoarthritis.

In addition to PHB1, PHB2 is also involved in inflammation. Indeed, heterozygous PHB2+/− were shown to be more sensitive to liver insults and inflammatory aggressions than wild-type animals, confirming a cytoprotective and anti-inflammatory role of PHB2 in liver (Sánchez-Quiles et al., 2012).

Lucas et al. (2013) recently discovered how PHB1 and PHB2 induce an immune response in B cells. The interaction of CD86 with PHBs was shown to induce the phosphorylation of IkBα, phospholipase Cγ2, and protein kinase Cε/δ(II), leading to the nuclear translocation of NF-κB (p65) into the nucleus and the subsequent transcription of Oct-2 and IgG1.

**PHBs and Metabolic Diseases**

Ande and Mishra (2009) and Ande et al. (2009a) established that PHB1 is a key regulator of insulin signaling and adipocyte differentiation. Indeed, PHB1 is directly phosphorylated by the insulin receptor to promote insulin signaling and block Akt signaling, whereas phosphorylation of PHB1 by Akt has the opposite effect.

Interestingly, PHB1 is not only phosphorylated but is also conjugated to O-linked β-N-acetylglucosamine in myoblast cells in response to insulin and high glucose. This glucose-induced O-GlcNAc modification and phosphorylation of PHB1 may be associated with insulin resistance modification and tyrosine phosphorylation of PHB (Ande et al., 2009b; Gu et al., 2011).

PHB1 inhibits pyruvate carboxylase and decreases insulin-stimulated oxidation of glucose and fatty acid in adipocytes, implying that it has a role in promoting lipid accumulation (Vessal et al., 2006). Crosslinking experiments showed that PHB1 associates with pyruvate carboxylase and Eps 15 homology domain protein 2 (EHD2), suggesting that PHB1 shuttles between the extracellular space and the mitochondria by a mechanism involving lipid rafts and EHD2.

Recently, Ande et al. (2012) demonstrated that treatment of preadipocytes with insulin or a peroxisome proliferator-activated receptor γ (PPARγ) agonist upregulates PHB1, thereby promoting preadipocyte differentiation into adipocytes. Remarkably, overexpression of PHB1 was sufficient to induce adipogenesis. PHB1 on the cell surface is also a marker of adipose vasculature and may be a possible therapeutic target for obesity (see below).

**PHBs and Pathogenic Agents**

Several recent studies demonstrated that the internalization of some viruses involves PHBs. For instance, PHB1 interacts with chikungunya virus (Wintachai et al., 2012), PHB2 interacts with dengue virus (Kuadkitkan et al., 2010), and both PHB1 and PHB2 interact with severe acute respiratory syndrome coronavirus (SARS-CoV; Cornillez-Ty et al., 2009). PHB1 and PHB2 also interact with the HIV-1 glycoprotein to promote replicative spread in nonpermissive cells (Emerson et al., 2010). Interestingly, recombinant capsid protein VP1 (rVP1) of foot-and-mouth disease virus dephosphorylates Akt and phospho-PPARγ, implicating PHB2 in host immune responses to infections.

**Drug Development Outlook**

The activities of PHBs are affected by some natural products (e.g., flavaglines and aurilide), fully synthetic small molecules (e.g., melanogenin), and adipotide, a chimeric peptide that is currently in phase I clinical trial. These compounds (except for adipotide) were examined for a variety of activities before it became clear that PHBs were their molecular targets. The best-studied PHB ligands are flavaglines.

**Flavaglines**

Flavaglines have a unique cyclopenta[b]benzofuran skeleton (Ebach et al., 2011; Ribeiro et al., 2012b). Rocaglamide, the first of these compounds to be described, was isolated more than 30 years ago by King et al. (1982) from medicinal plants of the genus Melia (Meliaceae) in Southeast Asia. Since then, ~100 other flavaglines, including rocaglaol and silvestrol, have been identified (Figure 3A; Figure S3).
Importantly, flavaglines did not display any overt sign of toxicity and inhibit cell proliferation.

PHB1 represses DNA replication by interacting with components of the mammalian replication machinery

References

Table 2. Roles of PHB Signaling in Cancer

| Antitumorigenic Roles of PHBs | References |
|------------------------------|------------|
| PHB1 activates the tumor suppressor p53 | Chander et al., 2011; Fusaro et al., 2003; Joshi et al., 2007 |
| PHB1 interacts with the tumors suppressors Rb, p107, and p130 to repress E2F-mediated transcription | Wang et al., 1999a, 1999b, 2002 |
| PHB1 represses DNA replication by interacting with components of the mammalian replication machinery | Rizwani et al., 2009 |

Protumorigenic Roles of PHBs

| PHB1 and PHB2 are necessary for the activation of C-Raf by Ras | Rajalingam et al., 2005 |
| phosphorylated PHB1 promotes the survival of prostate cancer cells | Zhu et al., 2010 |
| the oncomir miR-27a downregulates PHB1 | Liu et al., 2010; Fletcher et al., 2012 |
| localized on the cell surface, PHB1 mediates resistance to taxoids | Patel et al., 2010 |

Although flavaglines display a myriad of pharmacological effects due to their anticancer, anti-inflammatory, neuroprotective, and cardioprotective properties, it is the anticancer properties that have attracted the most attention from scientists (Ebada et al., 2011; Ribeiro et al., 2012b). At concentrations in the low-nanomolar range, flavaglines inhibit the proliferation of tumor cells, are not toxic to normal cells (Hausott et al., 2004; Ribeiro et al., 2012a; Su et al., 2006; Thuaud et al., 2008, 2011; Zhu et al., 2007), and show no sign of toxicity in mice (Bernard et al., 2011; Cencic et al., 2009; Lee et al., 1998; Thuaud et al., 2009; Zhu et al., 2009). King et al. (1982) were the first to demonstrate the antileukemic activity of rocaglamide in a mouse model. Subsequently, Ohse et al. (1996) established that flavaglines strongly inhibit protein synthesis, and Lee et al. (1998) demonstrated that these compounds delay the growth of human breast cancer cells in athymic mice. However, because the tumors were not eradicated, these investigations were interrupted. Since then, with the development of targeted therapies, approaches to the development of cytostatic compounds have changed radically (Gutierrez et al., 2009). As a consequence, there is now a renewed interest in flavaglines as potential anticancer agents.

Although flavaglines have shown significant anticancer effects in mouse models of cancer (Ainari et al., 2012; Cencic et al., 2009; Hwang et al., 2004; King et al., 1982; Lee et al., 1998; Lucas et al., 2009; Meurer-Grimes et al., 2002; Thuaud et al., 2011), their most promising potential is associated with their ability to enhance the in vivo efficacy of other anticancer drugs, and in particular to relieve the resistance of tumors to chemotherapy. Indeed, flavaglines were shown to enhance doxorubicin chemosensitivity in several mouse lymphoma models (Bordeleau et al., 2008; Cencic et al., 2009; Zhu et al., 2009). Importantly, flavaglines did not display any overt sign of toxicity in mice in these studies, consistent with previous observations.

Very recently, using affinity chromatography-coupled mass spectrometry, Polier et al. (2012) identified PHB1 and PHB2 as the direct targets of flavaglines. The binding of flavaglines to PHBs inhibits Ras-C-Raf-MEK-ERK signaling, and such signaling is essential for the survival of cancer cells (Figure 3B). C-Raf needs to interact with PHBs to be phosphorylated by Ras and thus participate in signaling (Rajalingam et al., 2005). The binding of flavaglines to PHBs prevents this interaction both in vitro and in vivo. Knockdown of either PHB1 or PHB2 with small interfering RNA (siRNA) mimicked the effects of flavaglines through inhibition of cap-dependent translation and an arrest of cell-cycle progression via a depletion of cyclin D3, CDK4, CDK6, and cdc25A. Moreover, rocaglamide strongly depleted PHB1 and phosphorylated C-Raf at the plasma membrane, suggesting that the relocation of PHBs to the membrane is involved in the mode of action of flavaglines. These various observations support the idea that flavaglines exert their anti-cancer effects through their action on PHBs.

These findings are of particular interest because we now have evidence that Ras-C-Raf-ERK constitutes an overactivated pathway in many human cancers, and that this pathway is a promising target for treatments in oncology (Matallanas et al., 2011; Maurer et al., 2011). Bleumink et al. (2011) showed that this inhibition of C-Raf-ERK signaling blocks cap-dependent protein synthesis. Indeed, ERK activates Mnk1, which phosphorylates eIF4E, allowing cap-dependent synthesis to occur. Few proteins require phosphorylated eIF4E for their synthesis, and most of them are involved in oncogenesis, angiogenesis, and chemoresistance. Thus, the in vivo anticancer effects of flavaglines seems to be a consequence of the inhibition of the synthesis of cyclins D1 and D3, CDK4, CDK6, cdc25A c-Myc, Bcl-2, survivin, Mcl-1, the PIM1/2 kinases, vascular endothelial growth factor (VEGF), and matrix metallopeptidase 9 (MMP9) (Bordeleau et al., 2008; Cencic et al., 2009; Nasr et al., 2013; Polier et al., 2012; Schatz et al., 2011).

Although inhibition of the Ras-C-Raf-ERK signaling pathway may be involved in the flavagline-induced suppression of cap-dependent synthesis in cells (Bleumink et al., 2011), it is more difficult to explain the observed inhibition of protein synthesis in vitro, where C-Raf, MEK, and ERK are absent. This observation suggests either that another molecular target is involved in the inhibition of translation by flavaglines or that PHBs interact with the translational machinery to control its activity.

Flavaglines induce apoptosis in cancer cells through a myriad of mechanisms (Ribeiro et al., 2012b) involving cytochrome c (Mi et al., 2006b; Zhu et al., 2007), apoptosis-inducing factor (Thuaud et al., 2009), caspase 12 (Thuaud et al., 2009), and the MAPKs JNK and p38 (Zhu et al., 2007, 2009). Whether these mechanisms involve PHB1 and PHB2 or another target remains to be determined. The effect of flavaglines on PHBs-interacting proteins other than C-Raf is also an issue that needs to be examined.

Silvestrol is the flavagline that has been the most studied in vivo for its anticancer properties (Ainari et al., 2012; Bordeleau et al., 2008; Cencic et al., 2009; Hwang et al., 2004; Kim et al., 2007; Mi et al., 2006a; Robert et al., 2009; Schatz et al., 2011). However, the development of this compound is severely limited by its sensitivity to P-glycoprotein-mediated multidrug resistance and its suboptimal absorption, distribution, metabolism, and excretion (ADME) characteristics (Gupta et al., 2011; Liu et al., 2012). Fortunately, structure-activity relationship (SAR)
studies have established that the deletion of the methyl ester or the amide in position 2 results in compounds that escape this multidrug resistance without any loss of cytotoxicity (Figure S3; Ribeiro et al., 2012a; Thuaud et al., 2009, 2011). In addition to their anticancer effects, flavaglines also display potent anti-inflammatory and neuroprotective activities. Indeed, flavaglines at nanomolar concentrations act as immunosuppressors by inhibiting the production of interferon-γ, TNF-α, IL-2, and IL-4 by T lymphocytes (Proksch et al., 2005). This immunosuppression is mediated by activation of the MAPKs JNK and p38, which selectively inactivate nuclear factor of activated T cells (NFAT; Figure 3C). It is still not known how flavaglines activate JNK and p38. At higher concentrations, flavaglines also inhibit NF-κB, another transcription factor that is involved in inflammation (Baumann et al., 2002). Interestingly, the synthetic flavagline IMD-019064 inhibits NF-κB signaling and displays potent in vitro anti-inflammatory effects that manifest as an inhibition of proinflammatory mediator release from astrocytes, microglia, and endothelial cells (Fahrig et al., 2005; Figure 3D). More importantly, IMD-019064 protects dopaminergic neurons both in vitro and in vivo in models of Parkinson’s disease (MPP+-induced neurotoxicity) and traumatic brain injury. Recent SAR studies led to the identification of FL40, which provides greater neuroprotection in vitro than IMD-019064 (Ribeiro et al., 2012a). The neuroprotective activity of flavaglines is currently being actively explored by the pharmaceutical company Intermed Discovery, which has been developing IMD-026259 (Figure 3A) for the treatment of Parkinson’s disease.

These studies have prompted evaluations of the cytoprotective potential of flavaglines in other tissues. The synthetic flavagline FL3 (Figure 3A), which displays a potent cytotoxicity in cancer cells (Thuaud et al., 2009), was shown to protect cardiomyocytes in vitro and in vivo against various stresses, including anthracycline cardiotoxicity (Bernard et al., 2011). The heart is extremely sensitive to the toxicity of anthracyclines, such as doxorubicin. These medicines are among the most effective anticancer drugs available and have antitumor activity against both hematopoietic and solid tumors; unfortunately, their clinical utility is markedly hampered by their cardiotoxicity, which can lead to dilated cardiomyopathy and congestive heart failure (Minotti et al., 2004).

FL3 protects cardiomyocytes from the apoptosis induced by both doxorubicin and serum starvation (Bernard et al., 2011). Interestingly, these stresses are of different natures: doxorubicin causes an oxidative stress, whereas serum starvation blocks the growth factor signaling that is necessary for cell survival, mimicking an important component of myocardial ischemia. Treatment with FL3 significantly reduces mortality and attenuates both apoptosis and fibrosis in the hearts of doxorubicin-treated mice. Thus, flavaglines may both enhance the anticancer efficacy of anthracyclines and alleviate their main adverse effect, cardiotoxicity. Anthracyclines are central to cancer chemotherapies, so the discovery of a new class of cardioprotective agents...
would be extremely valuable clinically, and further studies to examine this therapeutic application are therefore warranted.

The cardioprotective effects of flavaglines are mediated through the phosphorylation of the small heat shock protein Hsp27 (Figure 3E). Hsp27 is critical for protecting cells against many types of damage, including the cardiotoxicity of doxorubicin, and exerts its effects in several ways, including chaperone activity, control of redox homeostasis, and inhibition of apoptosis (Kostenko and Moens, 2009). Whether PHBs or another target is involved in this cardioprotective effect of flavaglines has not been examined.

Although the cytoprotective and anti-inflammatory effects of flavaglines have not yet been demonstrated to be mediated through a binding to PHBs, it is unlikely that another target is involved. Indeed, both their pharmacological effects and cytotoxicity in cancer cells occur at the same range of concentrations. As far as we know, no other natural product that possesses such a complex three-dimensional structure has been shown to bind to different classes of proteins with a comparably high affinity.

It would be useful to further evaluate this therapeutic application of flavaglines and elucidate the mechanisms involved. It may seem odd than an anticancer drug can also display various cardio- or neuroprotective effects; however, there are precedents. For example, rapamycin derivatives (Erlich et al., 2007; Khan et al., 2006; Malagelada et al., 2010) and HDAC inhibitors are both cardio- and neuroprotectants in addition to having anticancer effects (Bush and McKinsey, 2009; Mai et al., 2009). A first clue to understanding this paradox may be that PHB1 is localized mainly in the mitochondria of normal cells and in the nucleus in cancer cells (Chen et al., 2011). The translocation of PHB1 from the nucleus and cytosol to mitochondria promotes survival in normal cells but apoptosis in cancer cells (Fusaro et al., 2003). It is therefore tempting to suggest that the opposite behaviors of flavaglines in cancer and normal cells are the consequences of different intracellular localizations of PHBs.

Although many academic studies have demonstrated a therapeutic potential of flavaglines in preclinical models of cancers and inflammatory and cardiac diseases, these compounds have not yet been examined in a clinical trial. To our knowledge, only two pharmaceutical companies (Intermed Discovery and Infinity Pharmaceutical) are currently developing flavaglines; however, the clinical trials of these compounds have not yet been examined. To our knowledge, no other natural product that possesses such a complex three-dimensional structure has been shown to bind to different classes of proteins with a comparably high affinity.

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**Aurilide**

Isolated from the Japanese sea hare (Dolabella auricularia; Suenaga et al., 2004; Suenaga et al., 1996), aurilide is a cyclic depsipeptide that displays a strong cytotoxicity at nanomolar and subnanomolar concentrations on a panel of cancer cell lines (Figure 4A; Figure S4). Work involving affinity chromatography identified PHB1 as the molecular target of aurilide (Sato et al., 2011). The binding of this toxin activates the proteolytic processing of the dynamin-like GTPase optic atrophy 1 (OPA1), which in turn triggers remodeling of the mitochondrial cristae, leading to apoptosis. This devastating effect on mitochondria probably explains the extreme toxicity of this compound, which impedes its study in animal models and precludes its further development as a medicament.

**Melanogenin**

In a quest to identify compounds that modulate skin pigmentation, Snyder et al. (2005) screened a tagged-triazine library on unpigmented melanocytes (Figure 4B; Figure S5). They identified melanogenin as an inducer of pigmentation with an EC\textsubscript{50} of 2.5 \(\mu\text{M}\). This compound upregulates tyrosinase, the rate-limiting enzyme in the biosynthesis of melanin.

Snyder et al. (2005) conjugated melanogenin to an agarose support, which enabled them to identify PHB1 as the molecular target by affinity chromatography. Further biological investigations confirmed that PHB1 is responsible for the induction of pigmentation. Immunofluorescence microscopy studies showed that PHB1 was localized only in mitochondria, which led these authors to suggest that the binding of melanogenin to PHB1 may disrupt the interaction between PHB1 and a transcriptional factor, thereby causing its translocation to the nucleus and induction of the expression of tyrosinase, the rate-limiting enzyme in melanogenesis.

This study was the first to demonstrate and unravel the involvement of PHB1 in the regulation of mammalian pigmentation. Although these discoveries may provide a basis for the development of novel cosmetics and drugs for the treatment of pigment disorders, no further investigation in this direction has been reported.

Recently, Wu and Wu (2012) demonstrated that PHB1 is enriched in lipid rafts in HaCaT keratinocytes after UVB irradiation, and that this protects the cells against UVB-induced apoptosis. These findings are consistent with the notion that PHB1 contributes to protection of the skin against UVB.

**Adipotide**

Kolonin et al. (2004) and Staquicini et al. (2011) used phage display techniques to identify a peptide homologous to a region of annexin A2 that targets PHB1 at the surface of the vascular endothelial cells of white adipose tissue (Figure 4C). By fusion with a proapoptotic sequence, they obtained adipotide (CKGGRKDK-GG-DILAKLAKK), a chimeric peptide that damages the vasculature of the adipose tissue and consequently disrupts the blood supply to adipocytes. In obese mice, adipotide induced a substantial loss of weight and normalized metabolism, leading to a reversal of obesity without any adverse sign of toxicity. Surprisingly, food intake was reduced, suggesting that there is an unexpected relationship between the adipose tissue vasculature and the regulation of feeding behavior (Kim et al., 2010).

In obese rhesus monkeys, 4 weeks of adipotide treatment led to a 28% reduction in body fat and also reduced food intake and improved the animals’ metabolic status (i.e., reduced insulin resistance); these findings have implications for the development of treatment for type 2 diabetes (Barnhart et al., 2011). Adipotide is currently being developed by the biotech company Arrowhead Research Corporation, and entered a phase I trial involving obese patients with prostate cancer in July 2012. Because adipotide has a negative endocrine effect on tumor growth, it is expected to slow tumor growth in these patients.

**Capsaicin**

Capsaicin is a component of hot chili peppers that inhibits the proliferation of many cancer cell lines. Using affinity chromatography, Fletcher et al. (2012) demonstrated that capsaicin (0.1–1 nM) binds to PHB2, and that this binding induces
a series of proapoptotic events in human myeloid leukemia cells, including (1) the translocation of PHB2 from mitochondria to the nucleus; (2) the dissociation of PHB2 from Adenine Nucleotide Translocator 2 (ANT2), leading to a noncompetitive inhibition of ANT activity; and (3) the release of cytochrome c into the cytosol (Figure 4D). The latter two effects could be caused by the depletion of PHB2 in mitochondria, which would compromise mitochondrial integrity and promote apoptosis.

Capsaicin was used in these experiments at concentrations that are much too high to be therapeutically relevant, but this work may constitute the basis for an optimization program to develop a clinically useful drug.

**Sulfonyl Amidine Derivatives**

Sulfonyl amidine A inhibits the osteoclastogenesis involved in bone remodeling with an IC_{50} of 1.8 μM (Lee et al., 2010c; Figure 4E). Chang et al. (2011) used affinity chromatography to identify the molecular target involved in this process and found that this compound binds to PHB1 and three other proteins. Which of these proteins is the relevant target involved in this antiresorptive activity remains to be determined.

**Aftin-4**

While investigating how the toxic amyloid-β42 (Aβ42) peptide is produced in Alzheimer’s disease, Bettayeb et al. (2012) observed that the adenine derivative Aftin-4 promotes Aβ42 production in neurons (Figure 4F). Aftin-4 was found to interact with three proteins related to neural degeneration: PHB1, voltage-dependent anion channel 1 (VDAC1), and mitofilin.

**cHOxa**

The dihydrooxazole cHOxa, which displays some cytotoxicity with a GI_{50} of 7.2 μM in B16F0 cells, was shown to alkylate the Asp40 of PHB1, suggesting that it could be used as a chemical probe to analyze the structure of PHB1 (Trzeciakiewicz et al., 2011; Figure 4G).

**Conclusions**

The regulation by PHBs of cell survival, apoptosis, metabolism, and inflammation makes these proteins promising therapeutic targets for novel treatments for cancer and neurodegenerative, metabolic, and inflammatory diseases. Their intracellular localization and their translocation in response to proapoptotic signals differ substantially between normal and cancer cells. This divergence of behavior, which is not fully understood, may be exploited to develop therapeutic agents. The phase I trial of
adipotide for the treatment of obese patients with prostate cancer may be the first step on the road to developing PHB ligands of clinical value. The recent discovery that flavaglines target PHBs warrants further studies of this class of anticancer and cytoprotective agent. In addition to the ongoing exploration of the roles of PHBs in physiology and physiopathology, we anticipate that this field will stimulate research addressing other classes of PHB ligands with original pharmacological properties.

Over the last few years, PHBs have been found to interact with more than 60 other proteins to regulate a myriad of cellular events. There is no doubt that in the coming years, new partners and new cellular regulations will be uncovered. An important issue will be to determine when the alterations of PHB expression, subcellular localization, and signaling are involved in the etiology of not only cancers but also cardiac, neurological, inflammatory, and metabolic diseases. Translating this knowledge into clinical applications may lead to the development of new medicines and biomarkers for diagnosis and prognosis.

SUPPLEMENTAL INFORMATION
Supplemental Information includes five figures and one table and can be found with this article online at http://dx.doi.org/10.1016/j.chembiol.2013.02.006.

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