Front. Biol. 2010, 5(6): 516–523
DOI 10.1007/s11515-010-7700-5

REVIEW

Heme regulates protein homeostasis at transcription, protein translation, and degradation levels

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Abstract Heme, as a prosthetic group of proteins, is an iron-protoporphyrin involved in a wide range of cellular functions. Cellular heme levels vary due to the accurate balance of its synthesis and degradation. The "heme sensor protein" is currently a focus of investigation because heme has been found as a cellular signaling messenger involved in various biologic processes, including gene expression, protein localization, protein stability and microRNA processing. Several eukaryotic transcriptional factors can be regulated by heme, including heme activator protein (Hap1), Bach1, REV-erbα, and neuronal PAS domain protein 2 (NPAS2). Especially, the two circadian transcriptional factors serving as the heme sensor, REV-erbα and NPAS2, coordinate the circadian clock with metabolic pathways. It is well established that heme regulates the activity of heme-regulated eukaryotic initiation factor 2α (eIF2α) kinase (HRI), which serves as a feedback inhibitor of protein translation in both erythroid and non- erythroid cells. Additionally, heme is involved in protein degradation by inducing the degradation of several proteins such as the iron response regulator (Irr), iron regulatory protein 2 (IRP2), Bach1, and circadian factor period 2 (Per2). The N-end rule ubiquitin-dependent protein degradation pathway has also been identified as a sensor of heme, which blocks the function of arginyl-tRNA protein transferase (ATE1) and E3 ubiquitin ligase. In this review, we summarize the regulatory roles of heme at the levels of transcription, protein translation, and protein degradation, highlighting the role of heme in maintaining cellular homeostasis.

Keywords heme, transcription, protein translation, protein degradation

Received July 15, 2010; accepted September 6, 2010

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1 Introduction

Heme is a prosthetic group for some essential proteins in living cells, such as hemoglobin, myoglobin, cytochromes, catalase and so on, playing critical roles in oxygen transport, electron transfer, and catalysis. Heme is a protoporphyrin IX containing an iron atom in the center, in either the reduced ferrous (heme) or the oxidized ferric state (hemin). The iron in free heme is bound by four nitrogens of protoporphyrin IX. When heme binds to protein, the cysteine, histidine or tyrosine residue usually serves as the axial ligand of heme by coordinating the iron center of heme. In many five-coordinate heme binding proteins with one axial ligand, some specific gas molecules may bind to the distal position, such as O_2, nitric oxide (NO), or carbon monoxide (CO), while six-coordinate heme binding proteins with two axial ligands often behave as electron-transfer proteins (Hou et al., 2006).

Excess intracellular heme is highly toxic to cells, in part due to its oxidative damage and, therefore, the intracellular heme level is tightly controlled in living cells. Heme is synthesized through a highly conserved eight-step process in metazoans, with several critical enzymes involved. The first and the last three steps take place in the mitochondria, while all remaining steps occur in the cytoplasm (Severance and Hamza, 2009). The breakdown pathway for heme mainly involves heme oxygenase (HO), which is the rate-limiting step of heme catabolism. There are several HO isoforms existing in human cells. The expression and activity of the enzymes involved in heme synthesis and breakdown are strictly controlled by heme itself. Imbalance between synthesis and degradation leads to serious disease, such as porphyria (Furuyama et al., 2007). Although the pathway for heme biosynthesis has been well documented, the intracellular trafficking of heme remains poorly understood. Recently, Severance et al. (2010) identified 288 heme-responsive genes in *C.*

elegans, which provides insights into the regulation of heme homeostasis in metazoa.

Heme-regulatory motif (HRM) has been identified in diverse proteins and is thought to regulate the functions of proteins in response to heme binding. In this motif, the Cys-Pro dipeptide is absolutely conserved and is essential for heme binding, and there is a tendency for having a basic residue in the first position and a hydrophobic residue in the fourth position (Zhang and Guarente, 1995). HRMs mediate various functions of proteins by heme binding, such as protein translation (Chen, 2007), transcriptional regulation (Ogawa et al., 2001), subcellular localization (Suzuki et al., 2004), and protein degradation (Hu et al., 2008).

Besides being a stable prosthetic group, heme acts as a signaling molecule to serve diverse biologic functions. It was reported that DGCR8, which is essential for the first processing step of pre-miRNA, is also a heme-sensor protein. Dimerization induced by heme promotes its function in miRNA processing and maturation (Faller et al., 2007). Heme regulates the Ras-mitogen-activated protein kinase (MAPK) signaling pathway in some specific cells (Mense and Zhang, 2006). Glutamyl-tRNA synthetase (GluRS) and tryptophanyl-tRNA synthetases (TrpRS) were reported to bind heme, and their activities were regulated by this hydrophobic molecule (Levicán et al., 2007; Wakasugi, 2007). Especially, the identification of the role of heme in circadian rhythms has placed the iron porphyrin as the focus of much research (Raghuram et al., 2007; Yin et al., 2007; Yin et al., 2010). From the point of view of protein homeostasis, we highlight the regulatory roles of heme in transcription, protein synthesis, and degradation in this review.

2 Heme regulates transcription by binding to transcriptional factors

Heme binding to transcription factors is found in both prokaryotes and eukaryotes (Monson et al., 1992; Mense and Zhang, 2006; Raghuram et al., 2007; Yin et al., 2007). Here we focus on the regulation of heme on eukaryotic transcriptional factors. The yeast heme activator protein (Hap1) is the first example to be identified as a heme-sensitive eukaryotic transcriptional factor, which promotes transcription of genes required for respiration and for controlling oxidative damage (Zhang and Hach, 1999; Becerra et al. 2002; Lan et al., 2004). In the absence of heme, Hap1 is localized in the nucleus and is bound with other nuclear proteins to form a high molecular weight complex (HMC).The DNA binding activity of Hap1 is repressed in the complex and the transcriptional activity is inhibited (Zhang and Hach, 1999). When the intracellular heme level is elevated, the conformation of HMC is changed and Hap1 binds to DNA as a dimer with high affinity to activate transcription (Mense and Zhang, 2006). Hap1 contains seven HRMs; however, it was proved that only HRM7 is critical for heme activation of Hap1 and the other HRMs seem to play accessory roles for its activation (Mense and Zhang, 2006). It was also established that Hap1 directly represses its own transcription in a heme-independent fashion under hypoxic conditions. Because heme is synthesized only in the presence of oxygen, heme acts as a switch of Hap1 from a hypoxic transcriptional repressor to an aerobic activator (Hickman and Winston, 2007).

Bach1 is a transcription factor in mammalian cells. It forms a heterodimer with small Maf family proteins such as MafK, and binds to the Maf recognition element (MARE) region to inhibit the transcription of target genes, including heme oxygenase-1 (HO-1) and β-globin. Bach1 may function as a sensor of oxidative stress, the transcriptional activity of which can be regulated by heme and arsenite (Ogawa et al., 2001; Suzuki et al., 2004; Reichard et al., 2008). HO-1 is the rate-limiting enzyme involved in heme degradation, so Bach1 plays an essential role in maintaining the intracellular heme levels (Sun et al., 2002). Bach1 senses heme by multiple HRMs. Upon heme binding to the HRMs within the C-terminal region of Bach1, the DNA binding activity of Bach1-MafK heterodimers is markedly inhibited (Ogawa et al., 2001). It was also demonstrated that heme can induce the nuclear export of Bach1 through a novel heme dependent nuclear export signal (NES) on Bach1, which involves two HRMs (Suzuki et al., 2004). Therefore, heme regulates the transcriptional activity of Bach1 by diverse pathways. Recently, it was found that Bach1 forms a complex with p53 on a subset of its target genes to suppress the transcriptional activity of p53 by recruiting histone deacetylase 1 (Hdac1), thus inhibiting the process of cellular senescence. In view of the regulation of bach1 by oxidative stress and heme, Bach1 behaves as a negative regulator of p53 to connect oxygen metabolism and cellular senescence (Dohi et al., 2008).

The circadian clock plays an important role in controlling mammalian behavior and physiology, such as sleep-wake cycles, blood pressure, and energy metabolism. Although it is evidenced that circadian rhythms affect metabolism, the mechanism remains obscure at the molecular level. In recent years, the orphan nuclear receptor Rev-erbα has been identified as a principal regulator of the cyclic expression of Bmal1 (Guillaumond et al., 2005; Ripperger, 2006), which is an essential transcriptional activator controlling the expression of clock-related genes. Heme has been identified as a physiologic ligand to Rev-erbα and promotes REV-erbα to recruit the nuclear receptor co-repressor—NCoR-HDAC3 complex, resulting in decreased transcription of target genes, including Bmal1 (Fig. 1). Heme also suppresses the expression of some genes related to glucose metabolism (Raghuram et al., 2007; Yin et al., 2007). Therefore, Rev-erbα serves as a heme sensor to coordinate

the mammalian clock and energy metabolism (Yin et al., 2010). Interestingly, heme binding to Rev-erbα also leads to the repression of transcription of PPARγ coactivator-1 α (PGC-1α), a potent inducer of heme biosynthesis (Wu et al., 2009), which forms a feedback loop of heme homeostasis. The circadian clock and heme biosynthesis are reciprocally regulated.

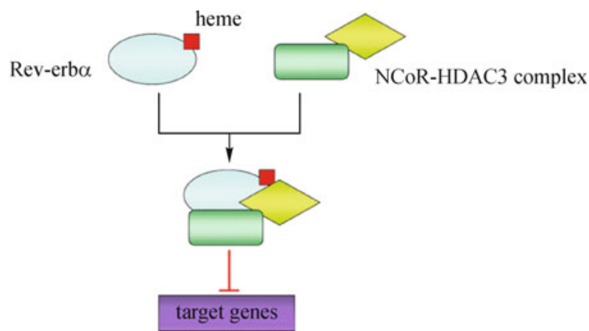

Fig. 1 Heme regulates gene transcription by binding to Rev-erbα. Heme-bound Rev-erbα can recruit corepressor NcoR-HDAC3 complex and then suppress the transcription of target genes, including Bmal1, PGC1-α and metabolic genes. PGC1-α: PPARγ coactivator-1 α.

Neuronal PAS domain protein 2 (NPAS2) is a core circadian gene, which forms a heterodimer with Bmal1 and is involved in the transcriptional regulation of circadian clock-related genes (Dioum et al., 2002; Hoffman et al., 2008). NPAS2 contains two PAS domains, each of which has a heme binding site. CO binding to the bound heme causes dissociation of NPAS2 from Bmal1 and then inhibits the DNA binding activity of the NAPS2–Bmal1 heterodimer. However, the other signaling gas— O_2 or NO- fails to react with NPAS2. CO is generated in heme catabolism catalyzed by heme oxygenase (HO), implying that the role of the NAPS2–Bmal1 heterodimer in circadian rhythms might be reciprocally linked to heme homeostasis (Dioum et al., 2002).

3 Heme controls protein translation by regulating HRI

Heme-regulated eukaryotic initiation factor 2α (eIF2α) kinase (HRI) regulates hemoglobin synthesis in erythroid cells by coordinating the synthesis of globin chains with heme availability (Chen and London, 1995). HRI specifically phosphorylates eIF2α at serine 51, blocks the GTP-GDP exchange required for the recycling of eIF2, and subsequently inhibits protein synthesis, behaving as a negative regulator of protein translation (Chen, 2007). HRI belongs to the family of the eIF2α kinases, which also include protein kinase R (PKR), GCN2, and protein kinase-like endoplasmic reticulum kinase (PERK). These kinases are regulated by diverse signals (Dever, 2002). The major regulator for HRI is heme in reticulocytes. Under normal conditions, heme binds to HRI and this binding leads to the inhibition of autophosphorylation of the kinase and promotes intermolecular disulfide bond formation. The kinase activity of HRI is inhibited, allowing ongoing synthesis of globin proteins. Under heme-deficient conditions, heme does not interact with HRI and the kinase is activated by autophosphorylation, which phosphorylates eIF2α at Ser-51 and blocks the protein translation (Fig. 2) (Chen and London, 1995). The eIF2α kinases share some degree of homology in their kinase domains. HRI contains five domains: N-terminal, kinase I, kinase insertion, kinase II, and C-terminal domains. The catalytic domain (kinase I and kinase II) is separated by a kinase insertion, which may be involved in dimerization (Chen and London, 1995).

Although the regulation of heme on HRI in erythroid cells has been established for a long time, the detailed molecular mechanism of heme sensing by HRI still remains unclear. Especially, the identification of a heme binding site has been a controversial issue even now. Chen's group proposed that HRI contains two distinct heme binding sites per monomer. One site located in the kinase insertion domain seems to bind heme dynamically and reversibly and regulates HRI kinase activity in response to the environmental heme concentration. Until now, the exact binding site has not been determined. Meanwhile, the other binding site is located at the N-terminal domain, which binds heme stably with the conserved His75 and His120 as the proximal and distal heme ligand, respectively (Fig. 2) (Rafie-Kolpin et al., 2000; Inuzuka et al., 2004). However, Shimizu's group proposed a different model to interpret the effect of heme on HRI. They hypothesized that only one heme binding site exists on the HRI monomer (Igarashi et al., 2008). It has been identified that heme binds to full-length HRI with two axial ligands. One is His-119 or His-120, located in the N-terminal domain, and the other one is Cys-409, situated in a Cys-Pro motif in the kinase insertion domain. It seems that heme behaves as a bridge to connect the N-terminal domain with the kinase domain. Under normal conditions, heme binds to HRI with the two axial ligands, leading to a closed and inactive conformation. While heme is deficient, the kinase domain is in an open and active conformation (Igarashi et al., 2008). It is pointed out that the proposed axial ligands His-119 and Cys-409 are conserved in mammals, and it is assumed that the heme-regulated HRI function is specific to mammals.

The kinase activity of HRI is also regulated by other environmental and chemical stimuli, such as nitric oxide (NO), heat shock, arsenite-induced stress, and so on (Chen, 2000; Igarashi et al., 2004). NO is a signaling gas molecule, identified as a second messenger, and plays a crucial role in various biologic processes such as apoptosis, neurotransmission, and cardiovascular homeostasis (Lamas et al., 2007). It has been reported that NO inhibits

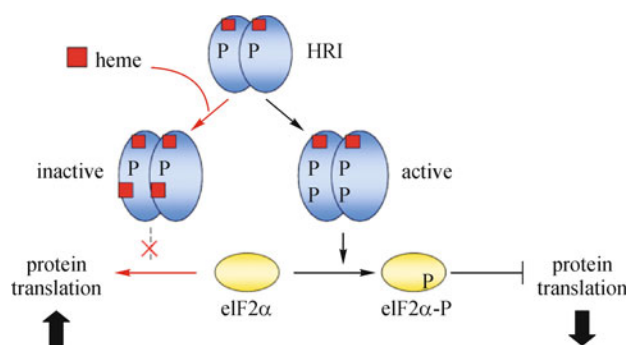

Fig. 2 Heme regulates protein translation by binding to HRI: two heme- binding site model. HRI monomer is synthesized with one heme molecule binding to the N-terminal domain stably. In heme deficiency, the kinase is activated by autophosphorylation. The active HRI phosphorylates eIF2α at Ser-51 and results in decreased protein translation. Under heme-abundant conditions, heme binds to the other binding site in kinase insertion domain and inhibits the kinase activity of HRI, allowing protein translation going on. HRI: heme-regulated eukaryotic initiation factor 2α (eIF2α) kinase.

protein synthesis by increasing eIF2α phosphorylation in eukaryotic cells (Kim et al., 1998). Later, it was identified that NO can activate the kinase activity of HRI by binding to the N-terminal heme binding domain (Uma et al., 2001; Igarashi et al., 2004). HRI is activated upon NO binding via heme (Igarashi et al., 2004). NO has high affinity for both protein-bound and free heme-iron (Tsai, 1994). HRI acts as a novel heme-based NO sensor protein by the formation of a five-coordinate NO-heme complex (Igarashi et al., 2004). The molecular mechanism of NO-induced activation of HRI remains obscure now. CO is also an important physiologic regulator in eukaryotic cells and it is the product in heme catabolism catalyzed by heme oxygenase. Like NO, CO binds to the N-terminal heme binding domain of HRI by forming a CO-heme complex but CO suppresses the activity of HRI and stimulates protein synthesis in rabbit reticulocyte lysate, and can reverse NO-induced activation of HRI (Uma et al., 2001). To clarify the mechanism of the opposite effects of NO and CO on HRI, more investigations will be required.

It is documented that heme-regulated protein synthesis is not restricted to erythroid cells, and HRI exists in numerous tissues and cultured cells, despite the abundance being low (Igarashi et al., 2004; Acharya et al., 2010). Very recently, HRI has been identified as a novel stress granule component to modulate protein synthesis initiation under stress conditions in Hela cells (Wehner et al., 2010). Heme-sensitive HRI has been detected in hepatocytes (Liao et al., 2007) and hepatic HRI is activated in acute heme-deficient states, resulting in translational shut-off of global hepatic protein synthesis (Liao et al., 2007; Acharya et al., 2010). In addition to acting as a heme-sensor to shut off protein synthesis, HRI normally modulates the basal hepatic endoplasmic reticulum (ER) stress tone and may be clinically relevant to acute hepatic porphyrias (Acharya et al., 2010).

In addition, the regulation of heme on HRI is multi-faceted. The HRI mRNA level in MEL or K562 cells increases upon induction of erythroid differentiation, which depends on the presence of heme. Heme may regulate HRI mRNA levels in addition to regulating its activity (Chen and London, 1995).

4 Heme is involved in protein degradation

As a prosthetic group, heme is crucial for stabilizing the protein structures in some cases. Heme controls the quaternary structure of neuronal NO synthase (nNOS) by inducing the subunit dimerizaiton. The heme-deficient nNOS is an inactive monomer, which is rapidly degraded in vitro (Dunbar et al., 2004). The heme ligand is also essential for the stability of hemoglobin (Gattoni et al., 1996). However, it has been established that HRMs are involved in protein ubiquitination and degradation. Protein degradation regulated by heme has been observed in both prokaryotes and eukaryotes. The bacterial iron response regulator (Irr) protein coordinates the heme biosynthetic pathway to prevent the accumulation of toxic porphyrin precursors under iron limitation (Hamza et al., 1998). The turnover of Irr is heme-dependent. Heme binds directly to an HRM near the N terminus, which is necessary for rapid degradation (Qi et al., 1999). Yang et al. further evidenced that a second heme binding site is also necessary for the turnover of Irr (Yang et al., 2005), and reactive oxygen species promote Irr turnover mediated by heme, suggesting that Irr oxidation is a signal for its degradation (Yang et al., 2005; Yang et al., 2006).

Iron regulatory proteins (IRPs) are key proteins in controlling iron homeostasis in mammalian cells. IRPs act as translational regulators to regulate the expression of the genes involved in iron uptake, use, storage and export by interacting with the iron responsive element (IRE) (Hentze et al., 2004). Up to now, two IRP isoforms, IRP1 and IRP2, have been identified, and their activities are regulated by different mechanisms. The protein level of IRP1 remains constant and its IRE binding activity is regulated by an iron-dependent Fe-S cluster assembly (Hentze et al., 2004). In contrast, IRP2 is rapidly targeted for degradation in iron-replete cells (Meyron-Holtz et al., 2004). The mechanism of the initiation of IRP2 degradation has not been fully characterized. It has been proposed that IRP2 degradation is triggered by heme binding to HRM, which has been suggested as playing a critical role in its recognition by heme-oxidized IRP2 ubiquitin ligase-1(HOIL-1), a RING finger ubiquitin ligase. Heme binding to HRM of IRP2 specifically contributes to its oxidative modification, which is considered a specific signal for polyubiquitination and proteasome-dependent degradation of the protein (Ishikawa

et al., 2005). Recently, the opinion has been challenged and other ubiquitin ligases have been identified (Salahudeen et al., 2009).

As mentioned above, heme regulates the transcriptional activity of Bach1 by inhibiting its DNA binding ability (Ogawa et al., 2001) and promoting nuclear export of Bach1(Suzuki et al., 2004). Furthermore, it was demonstrated that heme treatment induces polyubiquitination and rapid degradation of overexpressed Bach1 in several cell lines, and HOIL-1 may function as an E3 ubiquitin ligase. The three modes of regulation by heme show a cooperative effect at a system level to achieve a sensitive monitoring of heme levels inside cells (Zenke-Kawasaki et al., 2007).

A circadian factor period 2 (Per2) has a prominent role in circadian clock function. Heme has been identified as a prosthetic group of Per2, allowing the protein to sense redox status (Kaasik and Lee, 2004). A novel heme-regulatory motif within the C terminus of human Per2 (hPer2) has been identified as necessary for heme binding and protein destabilization. It was proposed that heme-mediated oxidation triggers hPer2 degradation, thus controlling gene transcription (Yang et al., 2008).

In 1980, it was shown that hemin specifically inhibits the energy-dependent degradation of normal and abnormal proteins in reticulocytes (Etlinger and Goldberg, 1980). Later, it was identified that hemin specifically inhibits the ATP-dependent ubiquitin-dependent proteolytic system of rabbit reticulocytes (Haas and Rose, 1981). It was proposed that hemin serves two opposing regulatory roles in controlling the accumulation of reticulocyte proteins *in vivo*. On one hand, hemin stimulates protein synthesis by inhibiting the activity of HRI; on the other hand, hemin inhibits ubiquitin-dependent protein degradation. Both effects favor the accumulation of proteins. It was documented that hemin also inhibits ubiquitin-dependent proteolysis in nonerythroid cells, being effective in extracts from a higher plant and in yeast, implying hemin regulation may be universal on ubiquitin-dependent proteolysis in all eukaryotic cells (Vierstra and Sullivan, 1988). However, it was reported that the hemin analog-protoporphyrin IX also inhibits ATP-dependent proteolysis, comparable to that of hemin. The molecular mechanism of hemin's effect on ubiquitin-dependent proteolysis remains to be clarified.

The N-end rule pathway of protein degradation is a ubiquitin-dependent proteolytic system, in which the N-terminal residues of proteins function as an essential component of the degradation signal, named N-degrons (Varshavsky, 1996). N-degrons contain basic residues and some bulky hydrophobic residues, which can be directly recognized by E3 ligase. While for those proteins with other residues at the N-terminal, N-terminal modifications are needed to convert these residues to N-degrons. N-terminal arginylation is a part of the N-end rule pathway and is a universal eukaryotic protein modification, playing an essential physiologic role in some biologic processes (Kwon et al., 2002; Brower et al., 2009; Leu et al., 2009; Kurosaka et al., 2010). Arginylation is catalyzed by arginyl-tRNA protein transferase (ATE1) by transferring Arg from Arg-tRNA^{Arg} to the N-terminal of target proteins with acidic residues at the N-terminal. Arginyl-tRNA synthetase (ArgRS) is indispensable in the N-end rule because it provides Arg-tRNA^{Arg} to ATE1 as its substrate. ArgRS is a very unique enzyme because it provides Arg-tRNA^{Arg} for both protein synthesis and protein degradation. Hu et al. demonstrated that the N-end rule pathway is a sensor of heme. Hemin inhibits the activity of ATE1 by inducing the formation of intramolecular disulfide bonds and induces the proteasome-dependent degradation of ATE1 in vivo (Hu et al., 2008). In addition, hemin binds to E3 ubiquitin ligase in the N-end rule pathway and blocks the function of E3 enzyme (Fig. 3). The N-end rule pathway is downregulated by hemin. ArgRS also takes part in the N-end rule pathway and our recent data showed that hemin could bind to human ArgRS and inhibited its catalytic activity *in vitro*, implying ArgRS might be regulated by hemin in the N-end rule pathway in human cells (Yang et al., 2010). Hemin regulates the N-end rule pathway in nonerythroid cells, further suggesting that the regulation of hemin on ubiquitin-dependent protein degradation may be general in all eukaryotic cells.

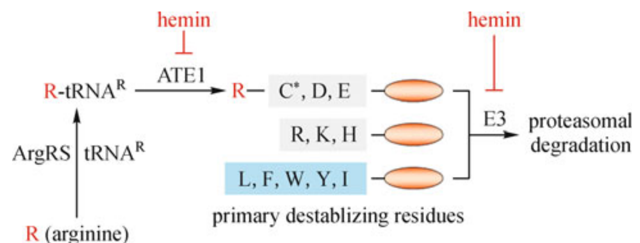

Fig. 3 A portion of N-end rule pathway. Hemin blocks the function of ATE1 and E3 ubiquitin ligase. ATE1: Arginyl-tRNA protein transferase; ArgRS: arginyl-tRNA synthetase.

5 Summary

The regulation of heme constitutes a very complicated network *in vivo*. Heme controls its own fate and at the same time governs cellular homeostasis. In this review, we focused on the regulatory functions of heme on cellular protein homeostasis from three levels — transcription, translation, and protein degradation. Heme plays an important role in protein quality control systems in mammals. However, other novel functions of heme have been explored. For example, hemin can interact with normal prion protein (PrP^c) and alter the subcellular localization of PrP^c, which might affect the function of PrP^c (Lee et al., 2007). Recently, it was reported that hemin inhibits cyclin D1 and insulin-like growth factor 1 (IGF-1) expression via a signal transducer and activator of

transcription 5B (STAT5b) under hypoxia in estrogen receptor α (ERα)-negative MDA-MB 231 breast cancer cells (Lim et al., 2010). It was also found that heme regulates a non-coding RNA in *Pseudomonas aeruginosa* (Oglesby-Sherrouse et al., 2010). It seems that the current knowledge about heme is just the tip of the iceberg and many of the intracellular functions might be still unknown. Therefore, more research is required to unveil other possible biologic roles of heme in the future.

Acknowledgements This work was funded by the National Key Basic Research Foundation of China (No. 2006CB910301), the National Natural Science Foundation of China (Grant No. 30930022), the Postdoctoral Science Foundation of China, the Postdoctoral Foundation of Shanghai, the Postdoctoral Foundation of Shanghai Institutes for Biological Sciences (SIBS), K.C. Wong Postdoctoral Fellowship of the Chinese Academy of Sciences, and Sanofi-Aventis-SIBS Postdoctoral Fellowship.

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