F-box and leucine-rich repeat protein 5 (FBXL5): Sensing intracellular iron and oxygen

Julio C. Ruiz, Richard K. Bruick *

Department of Biochemistry, University of Texas Southwestern Medical Center, 5323 Harry Hines Blvd., Dallas, TX 75390-9038, United States

A R T I C L E   I N F O
Article history:
Received 7 January 2014
Accepted 16 January 2014
Available online 25 January 2014

Keywords:
Iron
Oxygen
Reactive oxygen species
FBXL5
Iron regulatory proteins
Hemerythrin

A B S T R A C T
Though essential for many vital biological processes, excess iron results in the formation of damaging reactive oxygen species (ROS). Therefore, iron metabolism must be tightly regulated. F-box and leucine-rich repeat protein 5 (FBXL5), an E3 ubiquitin ligase subunit, senses intracellular iron and oxygen availability and regulates iron homeostasis by facilitating iron regulatory protein 2 (IRP2) degradation. FBXL5 possesses an N-terminal hemerythrin (Hr)-like domain that mediates its own differential stability by switching between two different conformations to communicate cellular iron availability. In addition, the FBXL5-Hr domain also senses O2 availability, albeit by a distinct mechanism. Mice lacking FBXL5 fail to sense intracellular iron levels and die in utero due to iron overload and exposure to damaging levels of oxidative stress. By closely monitoring intracellular levels of iron and oxygen, FBXL5 prevents the formation of conditions that favor ROS formation. These findings suggest that FBXL5 is essential for the maintenance of iron homeostasis and is a key sensor of bioavailable iron. Here, we describe the iron and oxygen sensing mechanisms of the FBXL5 Hr-like domain and its role in mediating ROS biology.

© 2014 Elsevier Inc. All rights reserved.

1. Introduction
Iron is the second most abundant metal in the Earth’s crust [1] and the most abundant in mammalian cells [2]. This abundance, coupled with the unique electrochemical properties stemming from its flexible coordination chemistry and wide range of reduction potentials [3], make iron an ideal cofactor for many essential biological processes. Iron is incorporated into numerous proteins, either directly or in the form of cofactors such as heme or iron-sulfur clusters and, is required for oxidative phosphorylation [4], oxygen transport [5] and DNA synthesis [6]. In addition, iron-containing proteins are involved in oxygen [7] and reactive oxygen species (ROS) sensing [8]. Therefore, iron is an indispensable nutrient in most living organisms.

Iron’s ability to easily gain and lose electrons also makes it potentially harmful. Excess iron can react with mitochondrial produced H2O2 and O2 leading to the formation of highly reactive hydroxyl radicals through the Fenton reaction [9,10]. Accumulation of reactive radicals creates conditions of oxidative stress, encountered in numerous pathological conditions such as in iron overload diseases (i.e. hemochromatosis) [9]. Thus, cellular iron homeostasis must be tightly regulated.

Cellular iron metabolism is regulated by the iron regulatory proteins (IRPs), which post-transcriptionally regulate the expression of genes involved in iron metabolism [11,12]. Since the activity of the IRPs is regulated in an iron- and oxygen-dependent manner [13–17], cells must be able to sense changes in the bioavailability of these metabolites. F-box and leucine-rich repeat protein 5 (FBXL5), an E3 ubiquitin ligase subunit, senses iron and oxygen through its N-terminal hemerythrin (Hr)-like domain and facilitates IRP2 degradation when iron is abundant [16,17]. In vivo disruption of FBXL5 expression results in inappropriate accumulation of IRP2 and unregulated iron uptake leading to iron overload and generation of damaging reactive oxygen species [16,18,19]. Therefore, FBXL5 plays an essential role in the maintenance of iron homeostasis.

2. Overview of iron homeostasis regulation
Mammalian cells have developed sophisticated molecular mechanisms to carefully regulate iron metabolism. Chief among these is the iron responsive element/iron regulatory proteins (IRE/IRP) system responsible for the maintenance of cellular iron homeostasis [12,20]. The IRPs are RNA-binding proteins that post-transcriptionally regulate, in a coordinated fashion, the expression of genes involved iron metabolism. IRPs recognize and bind to stem loop structures known as IREs located within the 5′ or 3′ untranslated regions (UTRs) of their target mRNAs [11,12,20]. Binding of the IRPs to an IRE located in the 5′ UTR (e.g. ferritin) attenuates the translation of the transcript [21] while IRP binding to IREs located within the 3′ UTR (e.g. transferrin receptor 1)
stabilize the target mRNA thereby promoting its expression (Fig. 1) [22]. Thus, IRPs allow cells to adjust the concentration of bioavailable cytosolic iron.

The RNA binding capacities of the IRPs are regulated in an iron-dependent manner. Under iron-deficient conditions, both IRPs are competent for mRNA binding. As iron bioavailability increases, IRPs lose their IRE binding capacity either due to Fe–S cluster assembly within IRP1 or the proteasomal degradation of IRP2 (Fig. 1) [13,16,17,23].

In addition to iron bioavailability, IRP stability and activity are regulated by oxygen [14,15,24]. Hypoxia stabilizes Fe–S clusters thereby diminishing IRP1’s RNA-binding capacity [25,26]. On the other hand, IRP2 stability and RNA-binding capacity are increased when oxygen is low [14]. Furthermore, it has been reported that excess ROS affect IRPs function, however, their mechanism of regulation is poorly understood [24,27]. Proper regulation of IRPs requires cells to be able to sense and respond to changes in iron and oxygen bioavailability.

The selective iron- and oxygen-dependent degradation of IRP2 is mediated by the Skp1/Cul1/Fbox (SCF) E3 ubiquitin ligase complex containing F-box and leucine-rich repeat protein 5 (FBXL5) (Fig. 2) [16,17]. Depletion of FBXL5 results in inappropriate accumulation of IRP2 under high iron conditions allowing aberrant expression of its target genes [16,18,19]. Interestingly, FBXL5 is regulated in a reciprocal manner to IRP2. FBXL5 is stabilized under iron- and oxygen-replete conditions (Fig. 2A) and polyubiquitinated to target it for proteasomal degradation when iron and oxygen are limiting (Fig. 2B) [16,17,28].

3. Iron and oxygen sensing by FBXL5 hemerythrin domain

Domain mapping revealed that regulatory elements conferring iron and oxygen responsiveness reside in FBXL5’s N-terminus. Bioinformatic and structural approaches showed that this region encodes a hemerythrin (Hr)-like domain [16,17]. Though this domain has been observed in proteins from prokaryotes and marine invertebrates, FBXL5-Hr domain constitutes the first Hr ever reported in mammalian cells.

These previously described hemerythrins often act as O2 reservoirs and transporters [29,30]. The structure of the domain is fairly simple. In canonical Hr domains, the polypeptide folds into a bundle of four α-helices. Buried in this helical bundle, there is a cavity filled with a metal complex. This complex consists of two iron ions, although other metals have been observed [31,32]. The di-iron center is bridged by two carboxylates coming from aspartate and glutamate residues and one solvent-derived hydroxide anion, resulting in the formation of a μ-hydroxo bridge (Fig. 3A) [33]. Three histidine nitrogens complete the coordination sphere of one iron atom; while the other iron atom is bound by only two histidine nitrogens, leaving it coordinately...
unsaturated (Fig. 3A) [34]. This feature is important for reversible dioxygen binding to a single iron atom. In oxy-Hr, an O₂ molecule can be reduced to peroxide while both iron atoms are oxidized to the +3 state. Only the 5-coordinately ion directly interacts with the O₂ molecule while the other iron ion acts as an electron reservoir [33,34]. A proton is transferred from the bridging hydroxo to the resulting peroxo species, which is stabilized by a μ-oxo bridge [29,30,34].

The FBXL5-Hr domain also adopts an α-helical bundle fold, which is stabilized by a di-iron center (Fig. 4A) [16,17,35–37]. However, unlike previously described hemerythrins, FBXL5’s Hr domain possesses several unique attributes. Unlike canonical hemerythrins, one of the iron coordinating histidines is replaced by a glutamate (Glu 58) residue. In addition, the iron atoms are bridged by two glutamate residues instead of a glutamate and an aspartate (Figs. 3B and 4B). The FBXL5-Hr domain contains an extra fifth helix packed against the apex of the canonical bundle that contributes a residue in the secondary coordination shell (Fig. 4A). It also has a truncated α-helix (helix 3) preceded by several disordered amino acids and an extended loop that contains an important regulatory element. Residues in this disordered region are part of a degron that mediates FBXL5’s stability [36]. In most Hr structures, each of the iron coordinating residues is located within a helix. However, in FBXL5-Hr one of the seven residues compromising the primary iron coordination shell is located within this partially disordered loop preceding helix 3 [35–37].

A bona fide sensor must undergo distinctive conformational changes or modify its activity upon fluctuations in the availability of the element being sensed. FBXL5, through its Hr domain, has emerged as a sensor of both iron and oxygen availability. In response to changes in iron bioavailability, FBXL5-Hr switches between two conformational states. In the presence of iron, this domain resides in a compact tertiary structure resistant to limited proteolysis that masks a degron comprised between residues 77–81 within the Hr domain itself; thus, promoting FBXL5 accumulation (Fig. 2A). When iron is limiting, assembly of the di-iron center is compromised. As a consequence, the Hr domain is destabilized as reflected by its decreased melting temperature and increased sensitivity to proteases both in vitro and in cultured cells [35]. Under these circumstances, the degron becomes accessible, and the protein is polyubiquitinated by an as yet unidentified E3 ubiquitin ligase and degraded by the proteasome (Fig. 2B) [35,36]. Consequently, iron uptake is promoted as IRP2 becomes stabilized.

The isolated FBXL5-Hr domain also responds to changes in oxygen availability. Oxygen deprivation promotes FBXL5 polyubiquitination and proteasomal degradation via its Hr domain [16,17,35–37]. However, no ordered electron density corresponding to a bound peroxo species was observed in any FBXL5-Hr crystal structures [35,36]. In addition, FBXL5-Hr expressed under oxidized conditions does not absorb light at 500 nm as is characteristic of the oxygen-bound form of canonical Hr proteins [35–37]. Inspection of FBXL5-Hr di-iron center reveals that it would be extremely difficult for O₂ to bind to the di-iron center. In the structurally homologous O₂-sensing DcrH Hr, there is a substrate tunnel that facilitates O₂ diffusion to the di-iron center. O₂ binding and subsequent oxidation of the diferrous complex causes a conformational change in a N-terminal loop that may promote further conformational changes in the entire protein [38]. Unlike DcrH Hr, the analogous O₂-binding site within FBXL5-Hr is occupied by bulky amino acids side chains that may decrease O₂ affinity through steric hindrance [35,36]. If FBXL5-Hr were to directly bind O₂, a substantial rearrangement of residues Phe-123 and Met-127 would be required to accommodate O₂ at the FeZ site. However, this has not been observed experimentally. Together, these data suggest that oxygen sensing is not mediated by direct oxygen binding to the di-iron center.

Even though FBXL5-Hr may not bind O₂ directly, it does contain a large solvent accessible surface near one of the Fe atoms that renders the oxidation state of the di-iron center sensitive to O₂ in the environment [35,36]. However, the precise mechanism by which oxidation of the di-iron center renders the degron increasingly susceptible to ubiquitination is unclear. A reduction in O₂ bioavailability may promote a subtle conformational change in the degron region not captured by

---

**Fig. 3.** Schematic representation of the di-iron center from deoxy-Hr and FBXL5-Hr domain. A, In canonical Hr domains the Fe atoms are bridged by an aspartate and a glutamate residue. Five histidines complete the coordination sphere of the Fe atoms. B, Among the unique features of the FBXL5-Hr is a glutamate (red, upper left side) that replaces a coordinating histidine. In addition, the iron atoms are bridged by two glutamate residues (blue, near center middle and lower parts).

Adapted with permission from Biochim Biophys Acta. 2012 Sep;1823(9):1484–90 (license # 3300850211726).
the analytical methods employed to-date. A crystal structure of a “reduced” FBXL5-Hr C159S mutant has been reported to lack the μ-hydroxo bridge between the two iron atoms [37], suggesting that the domain may become destabilized under these conditions. However, a subsequent unfolding of the domain under hypoxia, as might be expected upon significant rearrangement of the first coordination shell and iron loss, has not yet been experimentally observed [35,36].

The presence of a redox active di-iron center in the FBXL5-Hr domain could also facilitate regulation by reactive oxygen species. There are several examples in biology where iron-containing proteins act as ROS sensors. For instance, the transcription factor SoxR mediates the Escherichia coli response to superoxide. This transcription factor contains an Fe–S cluster that upon oxidation by superoxide induces a conformational change in the protein that results in increased expression of genes involved in superoxide catabolism [39,40]. In mammalian cells, IRP1 is a bifunctional cytosolic protein that uses an Fe–S cluster to determine iron availability and redox status [8,26,41]. Under iron replete conditions, IRP1 assembles a Fe–S cluster acquiring aconitase activity and losing IRE-binding capacity. The stability of the Fe–S is regulated by ROS and O2. Although its significance and mechanism is poorly understood, it is believed that ROS and O2 induce the oxidation of the Fe–S cluster promoting its disassembly from IRP1 [8,41]. Currently, there is no direct evidence suggesting that FBXL5 may play a role in ROS sensing. However, it has been reported that in cells treated with H2O2 or menadione, an oxidative stress generator, IRP2 is resistant to proteasomal degradation even in the presence of iron. Conversely, antioxidants such as ascorbate and N-acetyl cysteine induce proteasomal degradation of IRP2 [27]. One possibility is that H2O2-dependent oxidation of iron from Fe2+ to Fe3+ may impede assembly of FBXL5 Hr di-iron center resulting in protein degradation and therefore, IRP2 stabilization. However, this hypothesis is untested and alternative pathways cannot be excluded.

4. Physiological role of FBXL5

In vivo studies have determined that FBXL5 is essential for embryonic development and confirmed that it plays an essential role in the maintenance of cellular and systemic iron homeostasis [18,19]. IRPs control intracellular iron levels by regulating the expression of genes involved in iron uptake, storage and export [12]. Regulation of IRPs activity is essential to maintain iron levels within physiological levels.

Maintenance of intracellular iron levels is essential for embryonic viability. Mice lacking FBXL5 die in utero due to unregulated IRP2 accumulation that leads to iron overload by promoting iron uptake and impeding iron storage [18,19]. Excess iron accumulation in the early placenta results in the generation of damaging ROS and increased cell death [18]. Interestingly, generation of Fe2+/Fe3+ FBXL5+/− mice rescues the embryonic lethality. However, this rescue is not observed when FBXL5 and IRP1 are simultaneously deleted indicating that IRP1 and IRP2 have distinct physiological roles [18,19]. Moreover, selective deletion of FBXL5 in the liver results in death due to acute liver failure caused by iron excess only in animals fed a high iron diet [18]. These data suggest that FBXL5 protects cells from iron overload and oxidative stress by monitoring the intracellular ferrous iron pool and negatively regulating IRPs.

FBXL5 also plays a major role in the maintenance of systemic iron homeostasis. The liver is regarded as the primary iron-sensing organ in the body. It is involved in regulating iron absorption and iron release from storage. In response to increased levels of circulating iron, the peptide-hormone, hepcidin is released from the liver [42]. Hepcidin regulates iron transport across the duodenal mucosa as well as the release of recycled iron from macrophages, a process mediated by the only known iron exporter in mammals, ferroportin. Hepcidin promotes ferroportin internalization and degradation, thus, preventing iron transfer from the duodenum into the bloodstream [43,44]. Liver specific FBXL5 KO mice have significantly increased serum iron levels due to down-regulation of hepcidin [18]. Interestingly, FBXL5 also plays a role in establishing IRP iron responsiveness to control iron absorption in the duodenum. Unlike wild type littermates that manifest decreased hematocrit and hemoglobin levels when fed a low iron diet, FBXL5 heterozygous mice maintain normal hematological values due to increased iron absorption [19]. The duodenum of FBXL5 heterozygous mice accumulate much higher levels of IRP2 when fed a low iron diet, promoting increased expression of the iron importer divalent metal transporter 1 (DMT1) [19]. These data revealed that intestinal FBXL5 has a unique iron sensitivity, which confers to the duodenum a privileged role in the maintenance of systemic iron homeostasis, although the basis for this difference remains unknown. Overall, these data suggest that FBXL5 is an iron sensor that is required to ensure appropriate regulation of IRPs and maintenance of both cellular and systemic iron homeostasis.

5. Conclusion

FBXL5, through its N-terminal Hr domain, functions as a sensor of cellular iron and oxygen. The mechanisms by which FBXL5-Hr senses changes in iron and oxygen bioavailability are mechanistically distinct. While fluctuations in cellular iron concentration results in substantial conformational changes that facilitate protein degradation when iron is low, changes in oxygen availability are not accompanied by similar large-scale changes, even though the protein is also polyubiquitinated and degraded when oxygen is scarce. The exact mechanism by which FBXL5 Hr domain senses changes in O2 availability has yet to be determined. Importantly, by closely monitoring iron and oxygen intracellular levels, FBXL5 protects cells from iron overload and damaging oxidative stress. In addition, the presence of a redox active di-iron center in FBXL5-Hr may facilitate direct regulation by ROS. In vitro and in vivo studies have confirmed that FBXL5 plays a key role in maintaining cellular and systemic iron homeostasis.

Abbreviations

FBXL5 F-box and leucine-rich repeat protein 5.
Hr hemerythrin.
IRP iron regulatory proteins.
ROS reactive oxygen species.
IRE iron responsive element.
Tfr1 transferring receptor 1.
SCF Skp1/Cull1/F-box.

Acknowledgments

We thank Thomas Scheuermann and Mariano Ruiz for assistance with figure preparation. R.K.B. is the Michael L. Rosenberg Scholar in Medical Research and was supported by a Career Award in the Biomedical Sciences from the Burroughs Wellcome Fund, the Robert A. Welch Foundation (I-1568), and the National Institutes of Health (HL102481).

References
