Molecular mechanisms of normal iron homeostasis

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Humans possess elegant control mechanisms to maintain iron homeostasis by coordinately regulating iron absorption, iron recycling, and mobilization of stored iron. Dietary iron absorption is regulated locally by hypoxia inducible factor (HIF) signaling and iron-regulatory proteins (IRPs) in enterocytes and systematically by hepatic hepcidin, the central iron regulatory hormone. Hepcidin not only controls the rate of iron absorption but also determines iron mobilization from stores through negatively modulating the function of ferroportin, the only identified cellular iron exporter to date. The regulation of hepatic hepcidin is accomplished by the coordinated activity of multiple proteins through different signaling pathways. Recent studies have greatly expanded the knowledge in the understanding of hepcidin expression and regulation by the bone morphogenetic protein (BMP) signaling, the erythroid factors, and inflammation. In this review, we mainly focus on the roles of recently identified proteins in the regulation of iron homeostasis.

Iron Absorption and Loss

Dietary iron absorption requires that iron traverse both the apical and basolateral membranes of absorptive epithelial cells in the duodenum to reach the blood, where it is incorporated into transferrin (Tf). The transport of non-heme iron across the apical membrane occurs via the divalent metal transporter 1 (DMT1), the only known intestinal iron importer. Dietary non-heme iron exists mainly in ferric form (Fe^{+3}) and must be reduced prior to transport. Duodenal cytochrome B (DcytB) is a reductase localized in the apical membrane of intestinal enterocytes and is a major, but most likely not the only, reductase. In parallel, iron is also absorbed as heme. The transporter responsible for heme uptake at the apical membrane has not yet been conclusively identified. Cytosolic iron in intestinal enterocytes can be either stored in ferritin or exported into plasma by the basolateral iron exporter ferroportin (FPN). FPN is most likely the only cellular iron exporter in the duodenal mucosa as well as in macrophages, hepatocytes and the syncytial trophoblasts of the placenta. The export of iron by FPN depends on two multicopper oxidases, ceruloplasmin (Cp) in the circulation and hephaestin on the basolateral membrane of enterocytes, which convert Fe^{+2} to Fe^{+3} for incorporation of iron into transferrin (Tf).

Intestinal iron absorption is tightly controlled and is dependent on body iron needs. Recent studies indicate that this process is accomplished by modulating the expression levels of DMT1, DcytB and FPN by multiple pathways. First, the hypoxia-inducible factor (HIF)-mediated signaling plays a critical role in regulating iron absorption. Two studies[1,2] show that acute iron deficiency induces HIF signaling via HIF-2α in the duodenum, which upregulates DcytB and DMT1 expression and increases iron absorption. A conditional knockdown of intestinal HIF-2α in mice abolishes this response. Secondly, iron regulatory proteins (IRPs) are essential for intestinal iron absorption. DMT1 mRNA has an iron responsive element (IRE) at the 3′UTR and is stabilized upon IRP binding. In contrast, FPN mRNA has an IRE at the 5′UTR and IRP binding inhibits translation. Specific intestinal depletion of both IRP1 and IRP2 in mice markedly decreases the DMT1 and increases FPN, resulting in the death of the intestinal epithelial cells.[3] The mice die of malnutrition within two weeks of birth, underscoring the importance of these proteins. These results demonstrate the critical role of IRPs in the control of DMT1 and FPN expression. A novel isoform of FPN lacking an IRE was recently identified in enterocytes.[4] This FPN isoform is hypothesized to allow intestinal cells to export iron into the body under low iron conditions. DMT1 also expresses multiple isoforms with and without 3′IRE. In addition to DMT1 and FPN, both HIF signaling and IRP1 activation are associated with the regulation of iron absorption.[5,6] HIF-2α mRNA contains an IRE within its 5′-UTR.[5] Under conditions of cellular hypoxia, HIF-2α is derepressed through the inhibition of IRP-1–dependent translational repression.[6] Thirdly and importantly, FPN protein is negatively regulated by hepcidin, a critical iron regulatory hormone predominantly secreted by liver hepatocytes and discussed in detail in "Central Role of Hepcidin in Iron Homeostasis."

Thus, intestinal iron absorption is coordinately regulated by several signaling pathways and is sensitive to hypoxia by HIF-2α, enterocyte iron levels by IRP/IRE and bodily iron levels by hepcidin.

Although iron uptake into the body is tightly controlled, iron loss does not appear to be regulated. Under normal conditions iron is excreted through blood loss, sweat, and the sloughing of epithelial cells. These losses amount to approximately 1 to 2 mg of iron per day. Under certain pathological states, Tf, and therefore iron, can be lost when the kidney fails to reabsorb proteins from the urinary filtrate. These proteinurea syndromes result from the lack of functional cubulin, megalin, or ClC-5.[7] Cubulin and megalin are protein scavenging receptors, whose function in the proximal renal tubule is the reuptake of nutrients from the urinary filtrate. ClC-5, a voltage-gated chloride channel, is required for the acidification of endocytic vesicles and the release of iron from Tf.

Iron Recycling

Under physiological conditions, about 25 mg of iron per day is consumed by immature erythrocytes in the bone marrow for heme biosynthesis. The recycling of heme-iron from senescent erythrocytes constitutes the main source of iron for erythropoiesis. Macrophages in the liver and spleen are responsible for recycling heme-iron from senescent erythrocytes. Hemoglobin-derived heme is catabolized by the cytosolic heme oxygenase-1 to release iron, which is subsequently exported into circulation by FPN. Heme can also be exported directly into circulation via the heme exporter, feline leukemia virus subgroup C receptor (FLVCR), on macrophage plasma membranes. FLVCR plays a critical role in the export of excess heme from immature erythrocytes and hepatocytes.[8] Plasma heme is scavenged and transported by hemopexin to hepatocytes for degradation. Moreover, the natural resistance-associated macrophage protein 1 (Nramp1) participates in the efficient recycling of iron in macrophage following erythrophagocytosis.[9] Nramp1, a closely related family member of DMT1, is a divalent metal transporter expressed exclusively within the late endosomal and phagolysosomal membranes of iron-recycling macrophages and other professional phagocytes. The induction of acute hemolytic anemia results in a significant decrease of Tf saturation and hematocrit in the Nramp1 knockout (*Nramp1*$^{-/-}$) mice, but not in strain-matched wild type mice. In chronic hemolysis, *Nramp1*$^{-/-}$ mice retain markedly increased quantities of iron within the liver and spleen. They exhibit more splenomegaly and reticulocytosis.[9] Thus, in macrophages, Nramp1 transports iron out of the phagocytic vesicle and FPN exports iron out of the cell into the circulation.

Central Role of Hepcidin in Iron Homeostasis

Shortly after the discovery of hepcidin in 2000, its key role in iron homeostasis was realized. Hepcidin is similar to defensins, which act as antimicrobials as part of the innate immune system. Like defensins, hepcidin is synthesized as a precursor protein and processed to a smaller form, which in the case of hepcidin is 25 amino acids. Also similar to defensins, hepcidin forms an amphipathic beta sheet and possesses four highly conserved disulfide bonds. Unlike defensins, whose purpose is to kill bacteria, hepcidin is not secreted onto epithelial exterior surfaces and only has weak bactericidal activity. In keeping with hepcidin's function as a hormone, it is secreted into the blood, where it acts systemically to bind to and downregulate FPN.[10] FPN is predominantly expressed in macrophages, enterocytes, hepatocytes, and the syncytiotrophoblasts of the placenta, where it regulates iron entry into fetal blood. Thus hepcidin negatively regulates the uptake of iron by Tf, the major iron transport protein in the blood. Since Tf is the major source of iron for hemoglobin synthesis by red blood cell precursors, increased hepcidin limits erythropoiesis and is a major contributor to the anemia of chronic disease.

Hepcidin binding to FPN triggers FPN degradation. The binding site of hepcidin maps to a 19 amino acid sequence in the exterior segment of FPN, between the seventh and eighth transmembrane region in the proposed topology of this iron exporter.[11] A recent study showed that upon hepcidin binding to FPN, Janus kinase 2 (Jak2) is activated, resulting in the internalization, ubiquitination and degradation of FPN.[12] Hepcidin resistance leading to iron overload results from a lack of hepcidin binding to FPN, in the case of the C326 substitutions, or failure of hepcidin to downregulate FPN, due to mutations of the N144 or P64 residues.[13] The corresponding mutant proteins export iron normally but are not downregulated by hepcidin. The N144 and P64 residues are predicted to be in the cytoplasmic domain of FPN. How they cause hepcidin resistance remains to be discovered.

Regulation of Hepcidin Expression in the Liver

Under physiological conditions, hepatic hepcidin expression is elegantly regulated by a cohort of proteins that are expressed in hepatocytes, including the hereditary hemochromatosis (HH) protein called HFE, transferrin receptor 2 (TfR2), hemojuvelin (HJV), bone morphogenetic protein 6 (BMP6), matriptase-2 and Tf. Hepcidin expression can also be robustly regulated by erythroid factors, hypoxia, and inflammation, regardless of body iron levels.

HFE/TfR2/Tf Regulation of Hepcidin Transcription

TFR2 is predominantly expressed in hepatocytes. Like the ubiquitously expressed transferrin receptor 1 (TfR1), TfR2 is a type-2 membrane protein with a cytoplasmic N-terminus and a single transmembrane spanning domain followed by a large ectodomain. The ectodomain and transmembrane domains of TfR2 are 45% identical to that of TfR1 in amino acid sequence. The cytoplasmic domains of the two receptors bear no similarity, other than that they both have tyrosine-based internalization motifs. Mutations in TfR2 cause a rare recessive form of HH, which leads to the hypothesis that TfR2 is involved in the sensing of body iron levels by binding iron-loaded (holo), but not iron-depleted, (apo) Tf. Mice lacking functional TfR2 have a somewhat more severe iron overload disease than mice lacking functional HFE.

Although HFE has been studied intensively, its function is only beginning to be elucidated. HFE, the hereditary hemochromatosis protein responsible for type 1 HH was cloned and crystallized over 12 years ago. The crystal structure of HFE resembles MHC1 molecules. Like MHC1 molecules, it forms a heterodimer with β2-microglobulin. Unlike MHC1 complexes it does not bind peptides because the groove formed by the α1 and α2 domains of HFE is too narrow and distorted to accept peptides. The most common mutation in HFE is a single nucleotide substitution resulting in a C to Y substitution at amino acid 282, which disrupts a critical disulfide bond. In cells transfected with the mutant construct, the C282Y HFE fails to fold correctly, does not interact with β2-microglobulin, is found predominantly in the endoplasmic reticulum, and does not traffic to the cell surface efficiently. The equivalent mutation in mice results in iron overload and lower hepcidin levels than wild-type mice with similar iron levels, implying that either the set point for iron regulation has changed or that the mice have a blunted response to iron sensing. The fact that the mice lacking HFE are more severely affected than mice homozygous for the equivalent C282Y mutation indicates that the loss of HFE function results in HH. The low tissue levels of HFE combined with the milder phenotype of iron overload in mice with the C to Y mutation compared with the HFE knockout mice argue against the idea that HFE misfolding and ER stress play a significant role in this form of HH.

The mechanism(s) by which HFE affects hepcidin transcription are beginning to emerge. Tf and HFE compete for binding to TfR1. TfR1 is hypothesized to sequester HFE. Thus, increased iron-loaded Tf levels would result in the release of HFE to interact with other proteins. Consistent with this idea, mice with a mutated form of TfR1, which has reduced binding to HFE but similar binding to Tf, have higher hepcidin levels and a slight reduction in serum Tf-saturation.[14] Mice expressing *Tfr1 (R654A),* which has reduced Tf binding but intact HFE binding, in addition to wild type TfR1, show higher liver and heart iron levels and lower hepcidin levels. Mice expressing a mutant *Tfr1 (L622A)*, which has reduced HFe binding, had higher hepcidin levels.[14] All of the data are consistent with the idea that sequestration of HFE by TfR1 leads to decreased signaling and hepcidin expression.

TfR2 also interacts with HFE in transfected mammalian cells.[15] The co-crystal structure of the ectodomains of HFE and TfR1 indicates that the α1- and α2-helices in HFE interact with the helical domain of TfR1.[16] The sites of interaction between TfR2 and HFE are quite different.[17] In contrast to the HFE/TfR1 complex, Tf does not compete with HFE for binding to TfR2. TfR2-TfR1 and HFE-HLA-B7 chimeras were generated to map the domains of the TfR2/HFE interaction. The TfR2 domains that interact with HFE map to the putative stalk and protease-like domains of TfR2 located between residues 104-250 and to the α3 domain of HFE, both of which differ from the TfR1/HFE interacting domains. These results indicate that despite the similarity in sequence between TfR1 and TfR2, the two proteins interact with different domains of HFE. Importantly, the differences in the HFE binding to TfR2 and TfR1 allow HFE/TfR2 and Tf to form a complex.

The observations that HFE binds to TfR2 and that mutations in TfR2 result in a rare form of HH (type 3 HH) with similar severity to that of HFE HH (type 1) suggest that HFE and TfR2 may be part of the same signaling pathway. Tf-induced release of HFE from TfR1 and would be expected to increase the association of HFE with TfR2 and to stimulate of hepcidin transcription. Hepatic cell lines or primary hepatocytes that express both HFE and full-length TfR2 respond to Tf treatment by increased transcription of hepcidin, whereas those lacking either HFE or TfR2 do not respond.[18] However, in both TfR2-deficient mice and $Hfe^{-/-}$ mice hepcidin levels are still regulated by increases and decreases in dietary iron, indicating that dietary iron influences more than just the HFE/TfR2-mediated signaling. In addition, Pietrangello and colleagues report that two siblings in a family with mutations in both HFE ($HFE^{C282Y/H63D}$) and TfR2 ($TFR2^{317X/317X}$) have a more severe, juvenile hemochromatosis form of HH than a sibling with only the $HFE^{C282Y/H63D}$ mutations.[19] These results imply that TfR2 may regulate iron homeostasis in part through interactions independent of HFE.

Other Functions of HFE in Iron Homeostasis.

HFE has a wider tissue distribution than TfR2, which appears to be restricted mainly to liver and to hematopoietic precursors. Thus the pleiotropic effects that HFE exerts

on iron homeostasis are independent of TfR2 in most tissues except the liver. Like in tissues, HFE expression in tissue culture cells can inhibit non-Tf– and Tf-mediated iron uptake or iron efflux from cells depending on the cell type. In other cell lines, it inhibits Tf-mediated iron uptake or non-Tf–mediated iron uptake in a cell-type specific fashion. HFE expression in macrophage cell lines and in HT29 cells, an intestinal cell line, inhibits iron efflux from cells. The mechanisms by which HFE influences iron uptake and efflux from cells are only beginning to be understood. In cells expressing Zip14, an iron and zinc transporter, HFE expression decreases Zip14 by increasing its degradation.[20] No direct interaction of HFE with any transporters has been reported to date.

HJV, BMP6, and Matriptase-2

HJV is a GPI-linked membrane protein encoded by the gene, *HFE2*. HJV is related to members of the repulsive guidance molecules (RGMa and RGMb), which are found in the developing brain. Homozygous or compound heterozygous mutations in *HFE2* result in a juvenile form of HH. Mutations in both *HFE2* alleles in mice ($Hjv^{-/-}$) result in a marked increase of iron deposition in the liver, pancreas and heart.[21-23] The severe suppression of hepcidin expression in juvenile hemochromatosis patients and in $Hjv^{-/-}$ mice indicates that HJV plays a central role in the regulation of hepatic hepcidin expression. HJV is highly expressed in skeletal muscle and the heart and at lower levels in the liver. Recent studies indicate that hepatic HJV regulates hepcidin expression through interacting with the bone morphogenic proteins (BMP), matriptase-2, a serine-protease related to extracellular matrix proteases, and neogenin, a receptor for RGMa.[24-27]

HJV is a co-receptor for BMP2, BMP4, BMP5, and BMP6 and enhances hepatic hepcidin expression by enhancing BMP signaling.[24,28] BMPs are a subfamily of cytokines that belong to the TGF-β superfamily. The BMP subfamily signals through one set of receptor-activated SMADs (SMAD1, SMAD5, and SMAD8). The receptor-activated SMADs form a heteromeric complex with SMAD4, the central mediator in TGF-β/SMAD signaling, which translocates from the cytoplasm to the nucleus to regulate gene expression. The importance of BMP signaling in the regulation of hepcidin expression is supported by studies in mice with liver-specific deletion of SMAD4, which show decreased hepcidin expression and severe iron accumulation in the liver as well as in other organs.[29] Two potential BMP-responsive elements critical for BMP and HJV responsiveness in both the distal and the proximal regions of the hepcidin promoter could explain the transcriptional regulation of hepcidin by BMPs.[30]

BMP2, BMP4, and BMP6 are endogenously expressed in liver cells.[31,32] However, BMP6 appears to be the key BMP in the regulation of iron homeostasis.[33-35] Studies of mice with different levels of body iron show that the gene expression of only BMP6 in the liver is positively correlated with the changes of hepatic hepcidin expression.[33] More importantly, BMP6 gene knockout in mice causes the marked suppression of hepcidin expression and severe iron overload,[34,35] similar to $Hjv^{-/-}$ mice. Therefore, BMP6 appears to be a major ligand for the activation of hepcidin expression.

HJV also interacts with matriptase-2. Matriptase-2 is a serine protease encoded by the gene, *TMPRSS6*, and is predominantly expressed in liver. It is a type II transmembrane protein that has a short cytoplasmic domain, a transmembrane domain and a large extracellular domain. Its extracellular domain contains a membrane-proximal SEA (sea urchin sperm protein, Enteropeptidase, Agrin) domain, two CUB (complete protein subcomponents C1r/C1s motif, urchin embryonic growth factor and BMP1) domains, three LDLRa (low density lipoprotein receptor class A) domains, an activation domain, and a C-terminal catalytic domain.[36]

The critical role of matriptase-2 in iron homeostasis was first reported in 2008 by Du et al,[37] showing that lack of the matriptase-2 catalytic domain in *mask* mice results in a marked increase of hepatic hepcidin expression as well as microcytic anemia. Further studies in cell lines transfected with mantriptase-2 demonstrated that matriptase-2 is a robust suppressor of hepcidin expression.[37] This finding was later confirmed by *Tmprss6* knockout studies ($Tmprss6^{-/-}$) in mice[38] as well as in zebrafish.[27] Interestingly, clinical studies have also linked the homozygous or compound heterozygous mutations in *TMPRSS6* to the onset of iron-refractory iron deficiency anemia (IRIDA).[39,40] These patients have high urinary hepcidin, which could explain their anemia. Sequence analysis of *TMPRSS6* in these patients reveals that mutations are not localized and occur not only in the catalytic domain, but also in the CUB and LDLRa domains.[39,40] These results imply that the entire extracellular domain of matriptase-2 is important for its function. In agreement with these results, matriptase-2 interacts with HJV and inhibits hepcidin activation by cleaving membrane HJV into fragments, which presumably abolishes the function of HJV.[27] Importantly, the interaction of matriptase-2 with HJV does not require its catalytic domain.[27] Therefore, the entire matriptase-2 extracellular domain, rather than only the catalytic domain, is functionally important.

HJV is also cleaved by furin, a proprotein protease, located predominantly in the trans-Golgi network. The interaction of HJV with neogenin is required for cleavage of HJV by

furin.[26,27,41,42] Both neogenin and furin are ubiquitously expressed. Studies in transfected cells indicate that the HJV/neogenin interaction at the plasma membrane is necessary for retrograde-trafficking, cleavage by furin and rapid release of HJV from cells.[43] The furin-mediated HJV release is negatively regulated by iron-loaded Tf and possibly non-Tf iron.[27,41,42] Consistent with these results, *in vivo* studies also indicate an acute phase increase of serum HJV in iron-deficient rats.[41] Soluble HJV is postulated to compete with membrane-bound HJV in hepatocytes for limited local levels of BMP and thus would negatively regulate hepatic hepcidin expression. In keeping with this idea, soluble HJV suppresses the induction of hepatic hepcidin expression by BMP6 both *in vitro* and *in vivo*.[35] Since HJV is highly expressed in skeletal muscle, which has no or low matriptase-2 expression, we hypothesize that the HJV release from skeletal muscle is mainly through furin-mediated cleavage and that hepcidin expression is positively regulated by iron levels in the body via Tf saturation by decreasing HJV cleavage. Taken together, these data indicate that HJV is a key player in the regulation of hepcidin expression. BMP signaling is modified a complex interaction of multiple proteins, which control HJV protein trafficking and proteolytic processing.

Erythroid Factors

Immature erythroid cells are the major consumers of iron in the body. Although the fact that elevated erythropoiesis increases iron absorption regardless of body iron loading has been known for a long time, the sensors of erythropoietic state are only beginning to be understood. Three proteins, erythropoietin (EPO), growth differentiation factor 15 (GDF15) and twisted gastrulation (TWSG1), participate in this process. EPO is a well-characterized hormone essential for erythroid differentiation. A direct connection is now established between EPO and the suppression of hepcidin expression in hepatocytes, which express EPO receptors (EPO-R).[44,45] EPO-R signaling is proposed to be controlled by the binding of transcriptional factor C/EBPα to the hepcidin promoter, since a significant decrease of C/EBPα binding to the hepcidin promoter is observed when cells are treated with EPO.[45] Consistent with this observation, EPO-induced erythropoiesis downregulates hepcidin expression even in the presence of the hepcidin expression activator lipopolysaccharides (LPS) or dietary iron loading. EPO suppression of hepcidin expression is mediated through the downregulation of signal transducer and activator of transcription 3 (Stat3) phosphorylation triggered by LPS and of Smad1/5/8 phosphorylation induced by iron.[46] It is not yet clear how much this interaction contributes to EPO regulation of hepcidin. Some studies suggest that the effect of EPO on hepcidin is mostly indirect and requires the intermediate response of the bone marrow to EPO.

GDF15 is a family member of TGF-β superfamily and is secreted by hemoglobinized erythroblasts during the final stages of erythropoiesis. Elevated serum GDF15 correlates with decreased hepcidin and increased iron absorption in patients with β-thalassemia,[47] congenital dyserythropoietic anemia type I,[48] pyruvate kinase deficiency,[49] or refractory anemia with ring sideroblasts.[50] All these patients have the defective erythroid expansion. *In vitro* studies demonstrate a suppressive effect of GDF15 on hepcidin expression.[47] Interestingly, a more recent study shows that GDF15 expression is negatively regulated by the intracellular iron levels independent of HIF and IRP activation.[51] These findings are of interest because they are consistent with previous proposals that erythropoiesis is positively linked to intestinal iron absorption and storage iron mobilization and that the erythroid factor dominantly suppresses hepcidin expression in spite of iron overload.

TWSTG1 is the newest erythroid regulator of hepcidin expression.[52] In contrast to GDF15, it is produced mainly by the immature erythroid precursors during the early stages of erythropoiesis. TWSTG1 suppresses the upregulation of hepcidin expression induced by BMPs. In thalassemic animals, a significant increase of Twsg1 is detected in the spleen, bone marrow, and liver. TWSG1 protein interferes with BMP-mediated hepcidin expression in hepatoma cells and is a prime candidate to dysregulate iron homeostasis in thalassemia syndromes.

Hypoxia

Hypoxia is another suppressor of hepatic hepcidin expression that can act independently of iron levels in the body. The HIFs play a vital role in this process. HIF protein levels are negatively regulated by iron and oxygen. In the presence of oxygen, the regulatory subunit is modified by iron-dependent prolyl hydroxylases. The modified HIF interacts with the von Hippel–Lindau factor and is subsequently targeted for degradation through the ubiquitin/proteosome pathway. Under hypoxia or following iron chelation, the prolyl hydroxylase activity is inhibited, resulting in the accumulation and translocation of HIF into nucleus. HIF binding to the promoter of hepcidin leads to the suppression of hepcidin expression in hepatocytes[53] and increased iron uptake to meet the erythropoietic demand.

Inflammation and the Localized Regulation of Hepcidin

Hepcidin also plays a central role in the anemia of chronic disease. The inflammatory cytokines, most notably interleukin-6 (IL-6), induce signaling and activation of Stat3, which binds to the promoter region of hepcidin to activate transcription.[54,55] Macrophages, in addition to hepatocytes, also express hepcidin upon stimulation of the

Stat3 pathway by IL-6, by LPS, or by the toll-like receptor 4 pathway after ingestion of microbes including *Pseudomonas aeruginosa* and group A *Streptococcus.*[56-58] Whether hepcidin release by macrophages produces systemic or localized effects at the site of inflammation or bacterial invasion remains to be determined. Inflammatory cytokines can also transcriptionally regulate individual iron-related proteins directly, thereby altering iron homeostasis (reviewed recently in Weiss[59]).

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