King's Research Portal

Document Version
Peer reviewed version

[Link to publication record in King's Research Portal](#)

Zinc induces hephaestin expression via a PI3K-CDX2 dependent mechanism to regulate iron transport in intestinal Caco-2 cells

Hanuma Naik Ramavath[1], Purna Chandra Mashurabad[1], Puneeta Singh Yaduvanshi[1], Shobi Veleri[1], Paul A Sharp[2], Raghu Pullakhandam[1]*

[1]ICMR-National Institute of Nutrition, Hyderabad, India
[2] Department of Nutritional Sciences, Kings College London, UK

Running title: *Zn induces Hephaestin expression via PI3K -CDX2 pathway*

*Address for correspondence:

Dr. Raghu Pullakhandam
Scientist E
Biochemistry Division
National Institute of Nutrition
Jamai Osmania
Hyderabad 500076
India
E-mail: raghu_nin2000@yahoo.com

Abbreviations: DMT1: Divalent metal ion transporter-1; HEPH: Hephaestin; CDX2: Caudal-related homeobox transcription factor-2; TPEN: N,N,N',N'-tetrakis(2pyridinylmethyl)-1,2-ethanediamine; LY294002, 2-(4-Morpholinyl)-8-phenyl1(4H)-benzopyran-4-one hydrochloride.

Abstract

Zinc stimulates intestinal iron absorption via induction of divalent metal ion transporter (DMT1) and hephaestin (HEPH). While the increase in DMT1 is mediated via a PI3K/IPR2 axis, the mechanisms of Zn-induced HEPH expression downstream of PI3K remain elusive. In the current study we probed the role of Caudal-related homeobox transcription factor-2 (CDX2) on Zn-induced HEPH expression. Zn treatment of Caco-2 cells increased CDX2 phosphorylation and HEPH protein and mRNA expression. siRNA-silencing of CDX2 inhibited Zn-induced HEPH expression. LY294002, an antagonist of PI3K inhibited Zn-induced phosphorylation of CDX2, and downstream HEPH expression. These results suggest that increased expression of HEPH in intestinal cells following Zn treatment is mediated via a PI3K-CDX2 pathway.

Keywords: Iron, Zn, PI3K, Hephaestin, CDX2, Caco-2 cells, iron transporters, siRNA

Introduction:

Regulation of whole-body iron (Fe) homeostasis is essential for health. Humans lack a defined excretory pathway to remove excess iron from the body and thus primary control of Fe homeostasis occurs through regulating the rate of intestinal Fe absorption. Fe absorption by intestinal cells is synchronized with Fe status of the host such that higher systemic Fe status inhibits intestinal Fe absorption whereas absorption is increased by iron deficiency [1]. Dietary non-haem Fe exists predominantly in the ferric (Fe^{3+}) form, however, reduction to ferrous (Fe^{2+}) is essential for intestinal absorption. Duodenal cytochrome B (DcytB), a ferric-reductase, converts Fe^{3+} to Fe^{2+}, which is then transported across the apical membrane of enterocytes by divalent metal ion transporter-1 (DMT1) [2,3]. In the intestinal cells, Fe is either stored in ferritin or is transported across the serosal membrane by ferroportin (FPN); re-oxidised to Fe^{3+} by hephaestin (HEPH) and attaches to transferrin for onward transport in plasma [4-7]. The rate of Fe release is regulated by body Fe status via hepcidin, the major circulating regulator of Fe metabolism, which binds to FPN and blocks Fe egress [8]. In addition, cellular Fe levels mediate translational regulation of Fe transport proteins (ferritin, DMT1, FPN1) via the Iron Regulatory Proteins 1 and 2 (IRP1 and IRP2) [4,5,9].

Diet is an important factor in determining the absorption of Fe and other minerals. Phytic acid, abundant in cereal- and pulse-rich vegetarian diets inhibits the absorption of both Fe and zinc (Zn), and thus deficiencies of Fe and Zn often coincide especially in communities depending on these diets [10]. Dietary interactions between Fe and Zn are well documented with each imparting regulatory effects on the absorption of the other metal [1]. Intriguingly, co-supplementation with Fe and Zn shows a greater improvement in Fe status in children than supplementation with Fe alone [11]. Further cross-sectional studies also demonstrate positive associations

3

between serum Zn and haemoglobin [1,12-15]. In agreement with this, we have previously demonstrated that treatment of intestinal cells with Zn increases the uptake and egress of Fe, and this is associated with elevated DMT1 and HEPH expression [16-20]. Moreover, a dietary Zn depletion/repletion programme leads to changes in intestinal Fe transporter expression in rats which are related to changes in Zn status [21]. At the molecular level we have demonstrated that Zn induces DMT1 expression and this is mediated by IRP2-dependent mRNA stabilization [16]. Antagonists of PI3K inhibited the Zn-induced expression of DMT1 and HEPH in intestinal cells [16,17]. Intriguingly, HEPH mRNA has no specific consensus sequences for IRPs [22], indicating that the mechanism for HEPH regulation by Zn is distinct from that controlling DMT1 expression.

The intestine-specific homeobox transcription factors CDX1 and CDX2 have important regulatory roles in intestinal development, particularly proliferation and differentiation [23-25]. CDX2 is predominant in the crypt–villus epithelium of the small intestine and colon [24] and activates many genes required for cellular functions in enterocytes [23,26-28]. Upregulation of CDX2 inhibits intestinal cell proliferation [29], whereas mice with heterozygous deletion of CDX2 develop multiple polyps, in which epithelial cells appears to lack features of differentiation, implying a pivotal role of CDX2 in intestinal differentiation [30]. Chromatin immuno-precipitation studies identified CDX2 binding sites on *HEPH* promoter and silencing of CDX2 down-regulates HEPH expression [31,32]. Therefore, the purpose of this study was to test the hypothesis that Zn-induced HEPH expression in intestinal cells is mediated via CDX2.

Materials and Methods:

Materials*:* The primary antibodies against anti-CDX2 and p-AKT were procured from Cell Signalling Technologies (MA, USA). The anti-hephaestin primary antibody was

purchased from Santacruz Biotechnology (CA, USA). MG132 was bought from Tocris Bioscience (MO, USA). The antibiotic-mycotic mix and trypsin-EDTA were from *Invitrogen* (CA, USA). OptiMEM was purchased from Life Technologies, Paisley, UK. Minimum Essential Medium (MEM) (with glutamine), fetal bovine serum (FBS), LY294002 in solution, anti-β-actin antibody, and all other reagents were procured from Sigma Chemical Co. (Bangalore, India), unless specified in the text.

Methods:

Caco-2 cell culture: The Caco-2 cells (HTB-37) were purchased from ATCC (Rockville, MD, USA) and were cultured as previously described [16,20]. Briefly, the cells were cultured in 6-well plates with complete medium (MEM with 10 % (v/v) FBS, and 30 mmol/L penicillin/17.2 mmol/L streptomycin), at 37°C in an atmosphere of 5 % CO_2 and 95 % humidity. The complete medium was replaced every 2-3 days. When cultures reached 70 - 80 % confluence they were passaged using trypsin (0.25 %, w/v) in 1 mM EDTA. For the experiments, cells were allowed to undergo spontaneous differentiation for a period of 21 days, from the day of seeding.

Treatments: On day prior to the treatments, the medium was replaced with serum free MEM and the cells were incubated with 100 µmol/L $ZnSO_4$ (freshly prepared in MEM) or 2.5 µmol/L TPEN (prepared in DMSO) for a period of 0-24 h. Where required, LY294002 (25 µmol/L), MG132 (20 µmol/L), cycloheximide (100 µg/mL) or Actinomycin-D (8 µmol/L) were added 4 h prior to the Zn treatment.

Transient transfection of Caco-2 cells with siRNA: Gene knockdown by siRNA was performed as described previously [16,17]. Briefly, the medium was aspirated and the cells were rinsed once with pre-warmed Dulbecco's Phosphate Buffered Saline (DPBS). Subsequently, the cells were replenished with OptiMEM containing 5% FBS and allowed to equilibrate for 1 h. Then the cells were transfected with Lipofectamine 3000

containing either 100 nmol/L CDX2 siRNA (ID#S19046; Cat#4392420100, Thermo Fisher Scientific, USA) or a non-targeting scrambled siRNA (Santacruz, CA, USA), for a period of 72 h. Post treatment the medium was aspirated and the cells were washed with pre-warmed DPBS, and recharged with fresh OptiMEM containing 5% FBS for 42 hours. Prior to Zn treatments, the cells were once rinsed with pre-warmed DPBS and then incubated in serum free MEM for a period of 12-14 h.

Real time PCR: For analysis of gene expression the treated cells were harvested and total RNA was isolated using TRIzol and treated with DNase (AMPD1) for 10 min. RNA concentration and purity was assessed using a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, DE, USA). Samples were only used for analysis if the ratio of 260 nm/280 nm was >1.9. The RNA was reverse transcribed in to cDNA using commercial kit (iScript cDNA synthesis kit, #1708891, Bio-Rad, India) on a thermal cycler (Applied Biosystems, India). The transcript expression levels of *HEPH, CDX2* and *β-2 microglobulin* (internal housekeeping gene control) were analysed by real time PCR on a light cycler CFX 96 (Bio-Rad, India) using a SYBR Green PCR super mix kit (Bio-Rad, 1725271, India) The primer sequences of HEPH and β-microglobulin are given elsewhere[16,17]. The CDX2 (Gene bank accession No. NM_001354700.2) primer sequences targeting exon-1 were; forward primer 5'GACGTGAGCATGTACCCTAGC3'; reverse primer, 5'GCGTAGCCATTCCAGTCCT3'. All primers were procured from Eurofins, Bangalore, India. The target gene transcript level were normalized to the house keeping gene (β-microglobulin) and relative quantification was carried out using the $2^{-\Delta\Delta Ct}$ method. The data presented were normalised to the untreated control group in each experiment and were presented as the mean ± SEM.

Cell lysis and Immunoblotting: Following treatments, cell monolayers were washed thrice with 10 mmol/L PBS pH 7.2. The cells were lysed in Cell Lytic M reagent

supplemented with protease (P8340, Sigma) and phosphatase (P2850, Sigma) inhibitor cocktail (1X) and PMSF (1 mmol/L). The protein concentration was estimated by micro-BCA method and samples 20-40 μg were resolved on SDS-gels under reducing conditions. The separated proteins were transblotted onto nitrocellulose membranes. The membranes were blocked with 5% non-fat dry milk or bovine serum albumin (BSA) for 30 min, and were then incubated with respective primary antibodies (overnight at 4^oC). HRP-conjugated secondary antibodies were applied (1 h at room temperature) to detect the protein of interest. All the antibodies were diluted 1:1000, except anti-CDX2 which was diluted at 1:2000. For alkaline phosphatase treatments, the cell lysate (50 μg protein) prepared without phosphatase inhibitors was incubated over night with 6 units of alkaline phosphatase (from *Escherichia coli*) in 25 mM Tris-Cl buffer pH 7.2 containing 1 mM $MgCl_2$ and 1 mM $ZnCl_2$ at room temperature. The reaction was stopped by adding an equal volume of sample buffer. Then the sample was boiled and subjected to immunoblotting as described above. The proteins were detected using enhanced chemiluminescence detection kit (Bio-Rad, USA) and signals were acquired on a G-box imaging system (Syngene, USA). The blots were stripped and re-probed with anti-β-actin, the loading control. The signal densities on the blot were quantified using Image-J software (NIH, USA) and were normalized to the β-actin signal in corresponding lane.

Statistics: Statistical analysis was carried out using Sigma Plot (version 12, Systat Software Inc. IL, USA). All data are expressed as mean ± SEM. Data was analysed using one way analysis of variance followed posthoc least significant differences (LSD) test except that the mRNA of CDX2 and HEPH with TPEN treatment were compared by unpaired t-tests, and the level of significance was set at $p < 0.05$.

Results:

Our previous experiments demonstrated that Zn induces iron uptake and transport in differentiated Caco-2 cells with a maximal effect at 100 µmol/L [16] , and hence all the experiments were performed with this Zn concentration.

To determine whether Zn was modulating CDX2 expression in intestinal cells, we measured its mRNA and protein expression. Zn treatment significantly induced both CDX2 (a slower migrating form, as described below) and HEPH protein expression as a function of time (Fig.1). CDX2 protein expression peaked between 6- 8 h followed by a slight decline at 12 h, while HEPH protein expression was maximal between 6-12 h following exposure to Zn. As expected Zn also significantly induced the mRNA expression of HEPH, but not that of CDX2 (*supplementary figure 1*) compared to untreated control cells.

A closer observation of the CDX2 immunoblots revealed two specific bands in the region of 35-40 kDa in both control as well as Zn-treated cells (**Fig. 1**). The abundance of the slower migrating band was induced by Zn as a function of time whereas the faster migrating band was not. The presence of the slower migrating band could be due to phosphorylation of CDX2. To test this possibility, the cell lysate was treated with alkaline phosphatase (ALP). ALP treatment of cell lysates reduced the slower migrating form of CDX2 by ~85%, and pCDX2/CDX2 ratio was significantly lower in ALP treated cell lysates compared to respective controls, implying that slower migrating band was indeed a phosphorylated form of CDX2 (pCDX2) (**Fig. 2A**). Further, CDX2 protein abundance in the control and Zn treated cells remained similar after the ALP treatment. Since both CDX2 phosphorylation and HEPH expression were upregulated at 8h, all the subsequent experiments were conducted at this time point.

Next, we determined whether Zn treatment influences CDX2 mRNA translation. For this, translation was blocked by cycloheximide (CHX) prior to Zn treatment. Zn increased the abundance of p-CDX2, either in the presence or absence of CHX, while CHX alone had no influence on p-CDX2 levels (**Fig. 2B**). However, under the same conditions, Zn-induced HEPH protein expression was inhibited by CHX. It has been reported that p-CDX2 is degraded via proteosomal pathway [33]; therefore, we also tested the effect of the proteosomal inhibitor MG132 on CDX2 expression (**Fig. 2B**). MG132 increased p-CDX2 levels either in the presence or absence of Zn compared to the untreated control. Interestingly, HEPH was also significantly upregulated in MG132 treated cells. When intracellular Zn was depleted using the chelator TPEN, expression of total CDX2 (both phosphorylated and nonphosphorylated forms) and HEPH protein (**Fig. 3A**) and mRNA expression (**Fig. 3 B&C**) was decreased.

Zn-induced HEPH expression was blocked by actinomycin D, suggesting that the effect of Zn was at the level of *HEPH* gene transcription (supplementary figure 2). To investigate whether CDX2 mediated the transcriptional effects of Zn on HEPH we carried out siRNA knockdown of CDX2. Transfection with CDX2 siRNA led to a significant decrease in CDX2 expression compared to cells transfected with scrambled siRNA either in the presence or absence of Zn (**Fig. 4**). Interestingly, Zn treatment increased HEPH expression in the control, but not in CDX2 silenced cells (**Fig. 4**).

We have shown previously that Zn mediates its effects on HEPH expression via PI3K [17]. Therefore, we asked whether Zn-induced CDX2 phosphorylation was also mediated through PI3K. To test this, we used LY94002, an inhibitor of PI3K, in Caco-2 cells treated with or without Zn (**Fig. 5**). LY294002 treatment abrogated the Zn-induced CDX2 phosphorylation and this also resulted in concurrent reduction of HEPH

expression in Zn treated cells. As expected, LY294002 also blocked Zn-induced phosphorylation of Akt, which lies downstream of PI3K activation.

Discussion:

Epidemiological and clinical studies indicate an association between Zn with Fe status, with Zn being an independent predictor of hemoglobin levels [1]. We have previously demonstrated that Zn induces intestinal iron absorption *in vitro* and *in vivo* via modulating levels of DMT1 and HEPH [16-21]. The induction of DMT1 is mediated by PI3K dependent up-regulation of IRP2 [16], but HEPH lacks such specific iron responsive elements in its mRNA [34]. This suggests the existence of an alternate mechanism for increased expression of HEPH by Zn. In the present study, we demonstrated that a novel PI3K-CDX2 regulatory axis mediated Zn-induced HEPH expression in intestinal cells.

Previous studies indicated that CDX2 was involved in regulation of HEPH [31,32], but there was no evidence for modulation of CDX2 expression and/or activity by Zn. Therefore, we first tested whether Zn modulates CDX2 and thereby alters HEPH expression. Zn increased the mRNA and protein levels of HEPH consistent with our previous study [17], but CDX2 mRNA remained unaffected by Zn. CDX2 immunoblots consistently showed a doublet with a slower migrating form which was specifically induced by Zn. Further, the relative abundance of slower and faster migrating forms of CDX2 also varied between experiments. Previous studies have also demonstrated multiple migrating forms of CDX2 on immunoblots, and the slower migrating form of CDX2 was found to be phosphorylated [33]. ALP treatment led to the disappearance of the slower migrating form, implying that this band was a phosphorylated form of CDX2. Treating cells with CHX to block mRNA translation abrogated Zn-induced HEPH expression, but not CDX2 phosphorylation, which implied that the Zn-stimulated

increase in CDX2 phosphorylation was not dependent on translation. Studies in Caco-2 cells demonstrated that CDX2 expression was post-translationally controlled via a proteasomal pathway, and that phosphorylation was required for both its nuclear translocation and proteosomal degradation [35]. MG132, an inhibitor of the proteosomal pathway, increased the abundance of p-CDX2 either in the presence or absence of Zn. Interestingly, the increased abundance of p-CDX2 in the presence of MG132 but in the absence of Zn was also associated with an increase in HEPH expression, further suggesting that p-CDX2 is directly involved in the regulation of HEPH expression. Cellular Zn depletion with the chelator TPEN led to down regulation of both CDX2 and HEPH mRNA and protein. These results together suggest that CDX2 expression and activity is sensitive to cellular Zn status and this in turn regulated HEPH expression.

In previous studies, increased CDX2 and HEPH levels were correlated with enterocyte differentiation [35,36]. To probe the link between CDX2 and HEPH further, we carried out siRNA silencing of CDX2. CDX2-silencing significantly reduced CDX2 protein expression, including p-CDX2, and levels remained suppressed in the presence of Zn. Furthermore, HEPH expression was not stimulated by Zn in CDX2-silenced cells. In agreement with these findings, it has been demonstrated that HEPH levels correlate with CDX2 in mouse and human tissues, and that ectopic expression of CDX2 enhances while CDX2 silencing down-regulates HEPH expression in colonic cell lines [31]. Moreover, multiple CDX2 binding sequences have been identified in the in 5' flanking regions of *HEPH* gene, some of which transcriptionally activated a HEPH reporter gene construct [31,32]. These results together suggest that CDX2 transcriptionally regulates the HEPH expression.

We have previously demonstrated that Zn stimulated HEPH expression through the PI3K pathway via PKB/AKT [17]. In the present study, the PI3K inhibitor LY294002 in addition to blocking Zn-induced HEPH expression also blocked phosphorylation of both AKT and CDX2, indicating that CDX2 could be a downstream target of the PI3K/AKT pathway. The PI3K/AKT signalling pathway is utilized in proliferation, cell survival and growth in response to extracellular stimuli [37]. Although there is no direct evidence for the regulation of CDX2 by PKB/AKT, multiple studies have suggested a role for the PI3K or PTEN (phosphatase and tensin homolog deleted on chromosome 10, a negative regulator of PI3K), pathways on CDX2 activity. Independent studies found that PI3K activation stimulated intestinal cell differentiation [38] and that differentiation was associated with higher expression of both CDX2 and HEPH [35,36]. Therefore, it is feasible that differentiation-dependent up-regulation of CDX2 could be mediated via PI3K. In contrast, PTEN, has been shown to induce the expression of CDX2 and its DNA binding activity, while wortmannin, a PI3K antagonist, also induced CDX2 expression, which was shown to be functionally relevant in terms of modulating reporter gene activity [39]. Zn has also been shown to directly activate AKT phosphorylation via degradation or inhibition of PTEN activity [40,41]. These divergent observations of the effects of PI3K on CDX2 have been attributed to the differences in cell density, maturation or culture conditions [42]. Moreover, CDX2 target genes appear to differ between differentiated versus pre-confluent Caco-2 cells [43]. This latter point is relevant here as the present study was conducted in the differentiated Caco-2 cells (ideal model for assessing the regulation of iron homeostasis).

In summary above results suggest that Zn induces HEPH expression in intestinal cells via a PI3K- CDX2 axis, lending further mechanistic support to our previous *in vitro* and *in vivo* observations that intestinal cell Zn status is a strong modulator of Fe

absorption. These findings open the possibility to exploit Zn and/or PI3K pathway for modulating the Fe status, either in treating its deficiencies or overload.

Data availability statement: The data that supports the findings of this study are available in manuscript Figures 1-5 & and the online supplementary Figures of this article. For any additional information please contact the corresponding author (raghu_nin2000@yahoo.com) with a reasonable request.

References:

[1] P. Kondaiah, P.S. Yaduvanshi, P.A. Sharp, R. Pullakhandam, Iron and Zinc Homeostasis and Interactions: Does Enteric Zinc Excretion Cross-Talk with Intestinal Iron Absorption?, Nutrients 11 (2019). 10.3390/nu11081885.
[2] A.T. McKie, D. Barrow, G.O. Latunde-Dada, A. Rolfs, G. Sager, E. Mudaly, M. Mudaly, C. Richardson, D. Barlow, A. Bomford, T.J. Peters, K.B. Raja, S. Shirali, M.A. Hediger, F. Farzaneh, R.J. Simpson, An iron-regulated ferric reductase associated with the absorption of dietary iron, Science 291 (2001) 1755-1759. 10.1126/science.1057206.
[3] H. Gunshin, B. Mackenzie, U.V. Berger, Y. Gunshin, M.F. Romero, W.F. Boron, S. Nussberger, J.L. Gollan, M.A. Hediger, Cloning and characterization of a mammalian proton-coupled metal-ion transporter, Nature 388 (1997) 482-488. 10.1038/41343.
[4] P. Sharp, S.K. Srai, Molecular mechanisms involved in intestinal iron absorption, World J Gastroenterol 13 (2007) 4716-4724. 10.3748/wjg.v13.i35.4716.
[5] M.U. Muckenthaler, S. Rivella, M.W. Hentze, B. Galy, A Red Carpet for Iron Metabolism, Cell 168 (2017) 344-361. 10.1016/j.cell.2016.12.034.

[6] A.T. McKie, P. Marciani, A. Rolfs, K. Brennan, K. Wehr, D. Barrow, S. Miret, A. Bomford, T.J. Peters, F. Farzaneh, M.A. Hediger, M.W. Hentze, R.J. Simpson, A novel duodenal iron-regulated transporter, IREG1, implicated in the basolateral transfer of iron to the circulation, Mol Cell 5 (2000) 299-309. 10.1016/s1097-2765(00)80425-6.

[7] C.D. Vulpe, Y.M. Kuo, T.L. Murphy, L. Cowley, C. Askwith, N. Libina, J. Gitschier, G.J. Anderson, Hephaestin, a ceruloplasmin homologue implicated in intestinal iron transport, is defective in the sla mouse, Nat Genet 21 (1999) 195-199.

[8] H. Drakesmith, E. Nemeth, T. Ganz, Ironing out Ferroportin, Cell Metab 22 (2015) 777-787. 10.1016/j.cmet.2015.09.006.

[9] K. Pantopoulos, Iron metabolism and the IRE/IRP regulatory system: an update, Ann N Y Acad Sci 1012 (2004) 1-13. 10.1196/annals.1306.001.

[10] B. Lonnerdal, Dietary factors influencing zinc absorption, J Nutr 130 (2000) 1378S-1383S. 10.1093/jn/130.5.1378S.

[11] K. Alarcon, P.W. Kolsteren, A.M. Prada, A.M. Chian, R.E. Velarde, I.L. Pecho, T.F. Hoeree, Effects of separate delivery of zinc or zinc and vitamin A on hemoglobin response, growth, and diarrhea in young Peruvian children receiving iron therapy for anemia, Am J Clin Nutr 80 (2004) 1276-1282. 10.1093/ajcn/80.5.1276.

[12] R.S. Gibson, Y. Abebe, S. Stabler, R.H. Allen, J.E. Westcott, B.J. Stoecker, N.F. Krebs, K.M. Hambidge, Zinc, gravida, infection, and iron, but not vitamin B-12 or folate status, predict hemoglobin during pregnancy in Southern Ethiopia, J Nutr 138 (2008) 581-586. 10.1093/jn/138.3.581.

[13] C.R. Cole, F.K. Grant, E.D. Swaby-Ellis, J.L. Smith, A. Jacques, C.A. Northrop-Clewes, K.L. Caldwell, C.M. Pfeiffer, T.R. Ziegler, Zinc and iron deficiency and their interrelations in low-income African American and Hispanic children in Atlanta, Am J Clin Nutr 91 (2010) 1027-1034. 10.3945/ajcn.2009.28089.

[14] L.A. Houghton, W.R. Parnell, C.D. Thomson, T.J. Green, R.S. Gibson, Serum Zinc Is a Major Predictor of Anemia and Mediates the Effect of Selenium on Hemoglobin in School-Aged Children in a Nationally Representative Survey in New Zealand, J Nutr 146 (2016) 1670-1676. 10.3945/jn.116.235127.

[15] M. Folin, E. Contiero, G.M. Vaselli, Zinc content of normal human serum and its correlation with some hematic parameters, Biometals 7 (1994) 75-79. 10.1007/BF00205198.

[16] P. Kondaiah, M.F. Aslam, P.C. Mashurabad, P.A. Sharp, R. Pullakhandam, Zinc induces iron uptake and DMT1 expression in Caco-2 cells via a PI3K/IRP2 dependent mechanism, Biochem J 476 (2019) 1573-1583. 10.1042/BCJ20180939.

[17] P. Kondaiah, P.A. Sharp, R. Pullakhandam, Zinc induces iron egress from intestinal Caco-2 cells via induction of Hephaestin: A role for PI3K in intestinal iron absorption, Biochem Biophys Res Commun 523 (2020) 987-992. 10.1016/j.bbrc.2020.01.023.

[18] V. Iyengar, R. Pullakhandam, K.M. Nair, Iron-zinc interaction during uptake in human intestinal Caco-2 cell line: kinetic analyses and possible mechanism, Indian J Biochem Biophys 46 (2009) 299-306.

[19] V. Iyengar, R. Pullakhandam, K.M. Nair, Coordinate expression and localization of iron and zinc transporters explain iron-zinc interactions during uptake in Caco-2 cells: implications for iron uptake at the enterocyte, J Nutr Biochem 23 (2012) 1146-1154. 10.1016/j.jnutbio.2011.06.008.

[20] S. Yamaji, J. Tennant, S. Tandy, M. Williams, S.K. Singh Srai, P. Sharp, Zinc regulates the function and expression of the iron transporters DMT1 and IREG1 in human intestinal Caco-2 cells, FEBS Lett 507 (2001) 137-141. 10.1016/s0014-5793(01)02953-2.

[21] P. Kondaiah, R. Palika, P. Mashurabad, P. Singh Yaduvanshi, P. Sharp, R. Pullakhandam, Effect of zinc depletion/repletion on intestinal iron absorption and iron status in rats, J Nutr Biochem 97 (2021) 108800. 10.1016/j.jnutbio.2021.108800.

[22] G.J. Anderson, D.M. Frazer, A.T. McKie, S.J. Wilkins, C.D. Vulpe, The expression and regulation of the iron transport molecules hephaestin and IREG1: implications for the control of iron export from the small intestine, Cell Biochem Biophys 36 (2002) 137-146. 10.1385/CBB:36:2-3:137.

[23] M.A. Crissey, R.J. Guo, S. Funakoshi, J. Kong, J. Liu, J.P. Lynch, Cdx2 levels modulate intestinal epithelium maturity and Paneth cell development, Gastroenterology 140 (2011) 517-528 e518. 10.1053/j.gastro.2010.11.033.

[24] D.G. Silberg, G.P. Swain, E.R. Suh, P.G. Traber, Cdx1 and cdx2 expression during intestinal development, Gastroenterology 119 (2000) 961-971. 10.1053/gast.2000.18142.

[25] N. Gao, P. White, K.H. Kaestner, Establishment of intestinal identity and epithelial-mesenchymal signaling by Cdx2, Dev Cell 16 (2009) 588-599. 10.1016/j.devcel.2009.02.010.

[26] J. Park, S. Schulz, S.A. Waldman, Intestine-specific activity of the human guanylyl cyclase C promoter is regulated by Cdx2, Gastroenterology 119 (2000) 89-96.

[27] F. Boudreau, E.H. Rings, H.M. van Wering, R.K. Kim, G.P. Swain, S.D. Krasinski, J. Moffett, R.J. Grand, E.R. Suh, P.G. Traber, Hepatocyte nuclear factor-1α, GATA-4, and caudal related homeodomain protein Cdx2 interact functionally to modulate intestinal gene transcription implication for the developmental regulation of the sucrase-isomaltase gene, Journal of Biological Chemistry 277 (2002) 31909-31917.

[28] C. Domon-Dell, Q. Wang, S. Kim, M. Kedinger, B.M. Evers, J.N. Freund, Stimulation of the intestinal Cdx2 homeobox gene by butyrate in colon cancer cells, Gut 50 (2002) 525-529. 10.1136/gut.50.4.525.

[29] E. Suh, P.G. Traber, An intestine-specific homeobox gene regulates proliferation and differentiation, Mol Cell Biol 16 (1996) 619-625. 10.1128/mcb.16.2.619.

[30] K. Chawengsaksophak, R. James, V.E. Hammond, F. Kontgen, F. Beck, Homeosis and intestinal tumours in Cdx2 mutant mice, Nature 386 (1997) 84-87. 10.1038/386084a0.

[31] T. Hinoi, G. Gesina, A. Akyol, R. Kuick, S. Hanash, T.J. Giordano, S.B. Gruber, E.R. Fearon, CDX2-regulated expression of iron transport protein hephaestin in intestinal and colonic epithelium, Gastroenterology 128 (2005) 946-961. 10.1053/j.gastro.2005.01.003.

[32] M. Boyd, M. Hansen, T.G. Jensen, A. Perearnau, A.K. Olsen, L.L. Bram, M. Bak, N. Tommerup, J. Olsen, J.T. Troelsen, Genome-wide analysis of CDX2 binding in intestinal epithelial cells (Caco-2), J Biol Chem 285 (2010) 25115-25125. 10.1074/jbc.M109.089516.

[33] I. Gross, B. Lhermitte, C. Domon-Dell, I. Duluc, E. Martin, C. Gaiddon, M. Kedinger, J.N. Freund, Phosphorylation of the homeotic tumor suppressor Cdx2 mediates its ubiquitin-dependent proteasome degradation, Oncogene 24 (2005) 7955-7963. 10.1038/sj.onc.1208945.

[34] H. Chen, T. Su, Z.K. Attieh, T.C. Fox, A.T. McKie, G.J. Anderson, C.D. Vulpe, Systemic regulation of Hephaestin and Ireg1 revealed in studies of genetic and nutritional iron deficiency, Blood 102 (2003) 1893-1899. 10.1182/blood-2003-02-0347.

[35] J. Boulanger, A. Vezina, S. Mongrain, F. Boudreau, N. Perreault, B.A. Auclair, J. Laine, C. Asselin, N. Rivard, Cdk2-dependent phosphorylation of homeobox transcription factor CDX2 regulates its nuclear translocation and proteasome-mediated degradation in human intestinal epithelial cells, J Biol Chem 280 (2005) 18095-18107. 10.1074/jbc.M502184200.

[36] G.J. Anderson, S.J. Wilkins, D.M. Frazer, Y.M. Kuo, J.G. Gitchier, C.D. Vulpe, Subcellular localization and differentiation-associated expression of hephaestin: A protein required for intestinal iron absorption, Gastroenterology 4 (2000) A661.

[37] B.A. Hemmings, D.F. Restuccia, PI3K-PKB/Akt pathway, Cold Spring Harb Perspect Biol 4 (2012) a011189. 10.1101/cshperspect.a011189.

[38] P. Laprise, P. Chailler, M. Houde, J.F. Beaulieu, M.J. Boucher, N. Rivard, Phosphatidylinositol 3-kinase controls human intestinal epithelial cell differentiation by promoting adherens junction assembly and p38 MAPK activation, J Biol Chem 277 (2002) 8226-8234. 10.1074/jbc.M110235200.

[39] S. Kim, C. Domon-Dell, Q. Wang, D.H. Chung, A. Di Cristofano, P.P. Pandolfi, J.N. Freund, B.M. Evers, PTEN and TNF-alpha regulation of the intestinal-specific Cdx-2 homeobox gene through a

PI3K, PKB/Akt, and NF-kappaB-dependent pathway, Gastroenterology 123 (2002) 1163-1178. 10.1053/gast.2002.36043.

[40] W. Wu, X. Wang, W. Zhang, W. Reed, J.M. Samet, Y.E. Whang, A.J. Ghio, Zinc-induced PTEN protein degradation through the proteasome pathway in human airway epithelial cells, J Biol Chem 278 (2003) 28258-28263. 10.1074/jbc.M303318200.

[41] L.M. Plum, A. Brieger, G. Engelhardt, S. Hebel, A. Nessel, M. Arlt, J. Kaltenberg, U. Schwaneberg, M. Huber, L. Rink, H. Haase, PTEN-inhibition by zinc ions augments interleukin-2-mediated Akt phosphorylation, Metallomics 6 (2014) 1277-1287. 10.1039/c3mt00197k.

[42] J.P. Lynch, D.G. Silberg, To differentiate or proliferate? The interaction between PI3K/PTEN and Cdx2, Gastroenterology 123 (2002) 1395-1397. 10.1053/gast.2002.36374.

[43] M. Natoli, J. Christensen, S. El-Gebali, A. Felsani, P. Anderle, The role of CDX2 in Caco-2 cell differentiation, Eur J Pharm Biopharm 85 (2013) 20-25. 10.1016/j.ejpb.2013.03.018.

Figure Legends:

Figure 1. Effect of zinc on CDX2 and HEPH expression in Caco-2 cells: Differentiated Caco-2 cells were incubated with Zn (100 μmol/L) for 0 - 12h, after which the expression of total CDX2 (~35-40kDa, upper and lower bands) and HEPH (~150kDa) protein were measured. The bar graphs represent protein densities normalized to the respective housekeeping gene (β-actin; ~45kDa), from 3 independent experiments. Data are presented as the mean ± SEM. Bars that do not share a common superscript differ significantly, $p < 0.05$.

Figure 2: Effect of alkaline phosphatase, cycloheximide and MG132 on zinc-induced CDX2 expression. The control and Zn treated (8h) cell lysates were treated with alkaline phosphatase, followed by immunoblotting, and the ratio pCDX2 (upper band) and CDX2 (lower band) densities was presented (**A**). Differentiated Caco-2 cells were incubated with cycloheximide (CHX, 100 μg/mL) or MG132 (20 μmol/L) in the presence and absence of Zn (100 μmol/L) for a period of 8h, and the expression of CDX2 (~35-40kDa) and HEPH (~150kDa) was measured by immunoblotting (**B**). The bar graphs represent protein densities normalized to the respective housekeeping gene (β-actin, ~45kDa), from 3 independent experiments. *The CDX2 densities remained similar across the treatments in panel B (data not shown).* Data are presented as the mean ± SEM. Bars that do not share a common superscript differ significantly, $p < 0.05$.
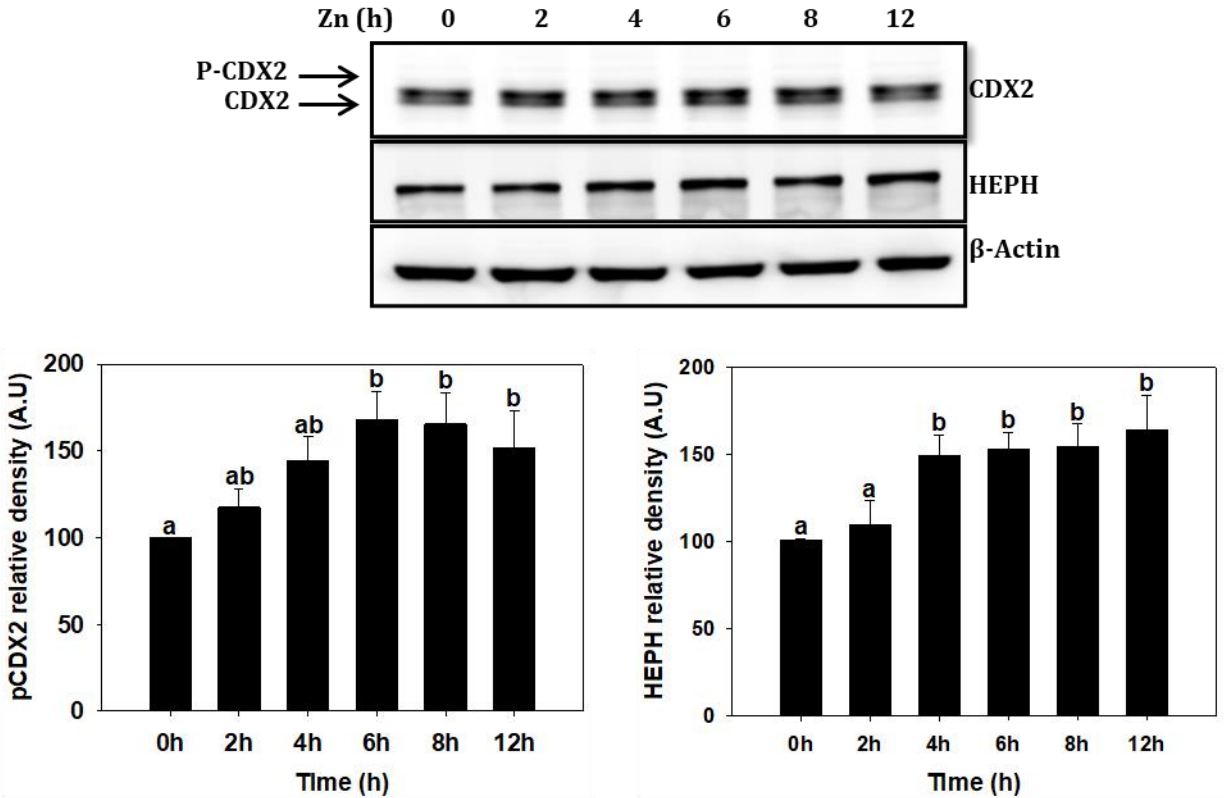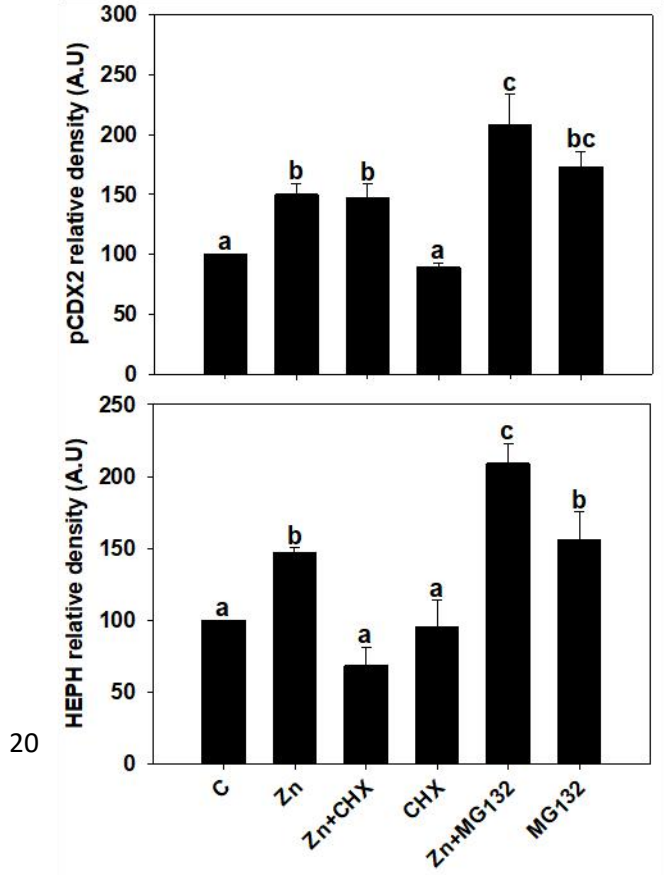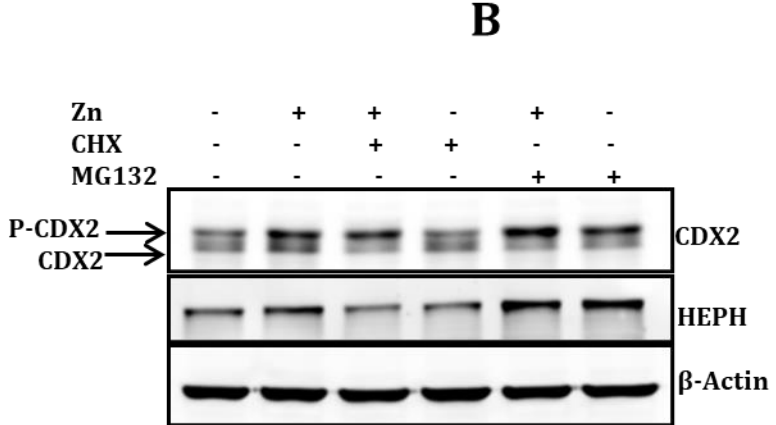
Figure 3: Effect of TPEN on CDX2 and HEPH expression: Differentiated Caco-2 cells were incubated with TPEN (2.5 μmol/L) for 6-24h, after which protein (A) and mRNA (**B, C**) expression of total CDX2 (~35-40kDa, upper and lower band) and HEPH (~150kDa) were measured. The bar graphs represent protein densities normalized to the respective housekeeping gene (β-actin, ~45kDa), from 3 independent experiments. The qPCR was performed in triplicate and repeated thrice to generate nine independent observations, and the data are normalized to the housekeeping gene, the β2-microglobulin. Data are presented as the mean ± SEM. Bars that do not share a common superscript differ significantly, $p < 0.05$. The expression of either CDX2 or HEPH are not statistically compared at 6h time point due to lower sample size (n=2).

Figure 4: Effect of CDX2 siRNA silencing zinc induced HEPH expression: Caco-2 cells grown in six-well plates were transfected either with CDX2 siRNA or Scrambled siRNA (control) and incubated in the presence or absence of Zn (100 μmol/L) for 8 h. The expression of phosphorylated (pCDX2, upper band) or nonposphorylated (CDX2, lower band) forms (~35-40kDa) and HEPH (~150kDa) was assessed by immunoblotting. The bar graphs represent protein densities normalized to the respective housekeeping gene (β-actin, ~45kDa), from 3 independent experiments. Data are presented as the mean ± SEM. Bars that do not share a common superscript differ significantly, $p < 0.05$.
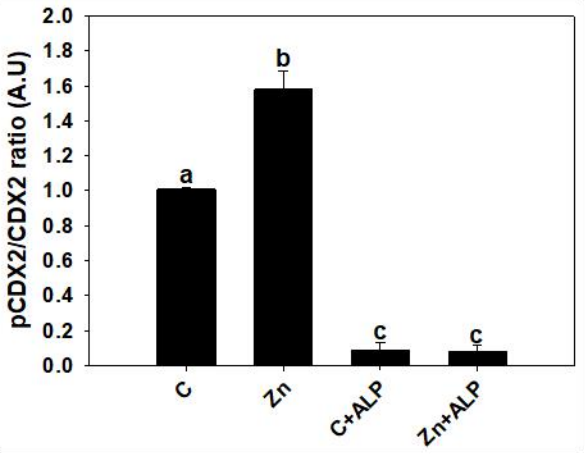
Figure 5. Effect of zinc and/or LY294002 on CDX2 and HEPH expression: Differentiated Caco-2 cells grown in 6-well plates were incubated with Zn (100 μmol/L)

and/or LY294002 (25 μmol/L) for 8h, after which the expression of CDX2 (~35-40kDa) and HEPH (~150kDa) was assessed by immunoblotting. The bar graphs represent protein densities normalized to the respective housekeeping gene (β-actin, 45 kDa), from 3 independent experiments. The densities of nonphosporylated CDX2 remained similar across the treatments (data not shown). Data are presented as the mean ± SEM. Bars that do not share a common superscript differ significantly, p<0.05.

Figure 1

Figure 2

A

B

20
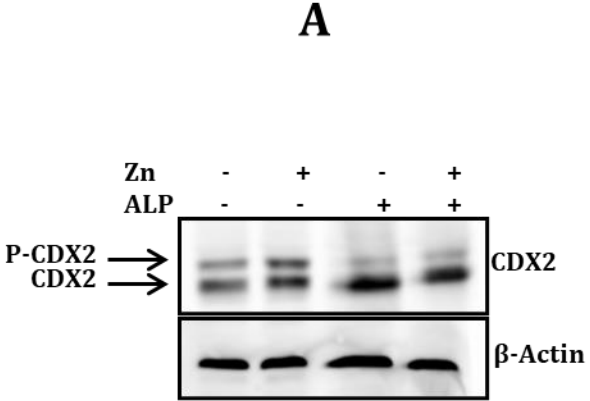
Figure 3

Figure 4

Figure 5