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1 **Zinc induces iron uptake and DMT1 expression in Caco-2 cells via a**
2 **PI3K/IRP2 dependent mechanism**

3
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9
10 **Running title:** *Zinc induces intestinal iron absorption*

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1 **Abstract:**

2 The absorption of dietary iron is influenced by numerous dietary and physiological
3 factors. We have previously demonstrated that zinc treatment of intestinal cells
4 increases iron absorption via induction of the apical membrane iron transporter
5 divalent metal iron transporter-1 (DMT1). To better understand the mechanisms of
6 zinc-induced iron absorption we have studied the effect of zinc on iron uptake, iron
7 transporter and iron regulatory protein (IRP 1 and 2) expression and the impact of
8 the PI3K pathway in differentiated Caco-2 cells, an intestinal cell culture model. We
9 found that zinc induces DMT1 protein and mRNA expression. Zinc-induced DMT1
10 expression and iron absorption were inhibited by siRNA silencing of DMT1. Further,
11 zinc treatment led to increased abundance of IRP2 protein in cell lysates and in
12 polysomal fractions, implying its binding to target mRNAs. Zinc treatment induced Akt
13 phosphorylation, indicating the activation of the PI3K pathway. LY294002, a specific
14 inhibitor of PI3K inhibited zinc-induced Akt phosphorylation, iron uptake, DMT1 and
15 IRP2 expression. Further, LY294002 also decreased the basal level of DMT1 mRNA but
16 not protein expression. siRNA silencing of IRP2 led to down regulation of both basal and
17 zinc-induced DMT1 protein expression, implying possible involvement of post-
18 transcriptional regulatory mechanisms. In agreement with these findings zinc treatment
19 stabilized DMT1 mRNA levels in actinomycin-D treated cells. Based on these findings,
20 we conclude that zinc-induced iron absorption involves elevation of DMT1 expression
21 via stabilization of its mRNA, via a PI3K/IRP2-dependent mechanism.

22

23 **Keywords:** Zinc, Iron, interactions, IRP2, PI3K, DMT1, Akt, intestine, Caco-2 cells

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1 **Introduction:**

2 Iron and zinc deficiencies coexist in populations subsisting on phytic acid-rich
3 vegetarian diets [1] suggesting that fortification and/or supplementation with both iron
4 and zinc should be considered to improve mineral status. However, some studies in
5 humans, animals and cell culture models have indicated competitive interactions
6 between iron and zinc at supplemental concentrations [2, 3]. Conversely,
7 epidemiological studies found a strong positive association of serum zinc with
8 haemoglobin levels [4, 5]. Furthermore, experimental zinc deficiency in rats leads to
9 development of anaemia and reduced erythropoiesis [6]. Interestingly, separate
10 delivery of iron and zinc leads to improved haematological responses compared to iron
11 supplementation alone in children [7]. These observations suggest that though iron and
12 zinc interact negatively when supplemented together, zinc may still have a positive
13 impact on iron status. These effects may be mediated either by enhanced intestinal iron
14 absorption or increased metabolic utilization of iron.

15 In the absence of obligatory excretory pathways, systemic iron homeostasis is
16 primarily regulated by modulating intestinal absorption [8]. The divalent metal ion
17 transporter-1 (DMT1), a proton-coupled metal ion transporter localized predominantly
18 in duodenum [9], mediates the intestinal iron absorption. At the apical surface of the
19 enterocyte, duodenal cytochrome B (DcytB) reduces ferric iron (Fe^{3+}) to ferrous (Fe^{2+})
20 [10] and facilitates its uptake via DMT1. Once absorbed, iron is either stored in ferritin
21 or exported across the serosal membrane through a ferroportin (Fpn1)- hephaestin
22 (HEPH)-mediated process [11, 12].

23 The cytosolic iron regulatory proteins 1 and 2 (IRP1 and IRP2), post-
24 transcriptionally regulate expression of iron metabolic proteins by binding to iron
25 responsive elements (IREs), stem loop structures, at the 5' or 3' untranslated regions

1 (UTRs) of target mRNA. Binding of IRPs to the 5' UTRs inhibits protein translation while
2 binding to 3' UTRs stabilizes the mRNA and thereby increases protein expression [13].
3 IRP1 is a bifunctional protein, which requires disassembly of a 4Fe-4S cluster for
4 activation. In contrast, IRP2 expression is inducible, depending on cellular iron levels,
5 and levels are controlled by proteosomal degradation. Induction of IRP2 expression
6 and/or activation of IRP1 during iron deficiency ensure increased iron absorption and
7 mobilization from intestinal cells. Interestingly, in addition to iron status, a variety of
8 stress conditions influence iron metabolism via IRP-dependent processes [14].

9 Previous studies in intestinal cells demonstrated that zinc treatment stimulates
10 the iron uptake and transcellular transport by inducing DMT1 and Fpn1 expression [15,
11 16]. Interestingly, zinc-induced Fpn1 expression has been shown to be mediated by
12 MTF1 in mouse fibroblasts [17]. Though initial studies identified MREs in 5' promoter
13 region of DMT1, latter studies ruled out such possibility [18] and therefore the
14 mechanism of zinc induced DMT1 expression and iron absorption remained elusive.

15 Zinc has gained interest as a potent cell signalling mediator [19-23]. Zinc ions
16 have been shown to activate numerous signalling pathways involving the receptor or
17 non-receptor tyrosine kinases, Ras/mitogen-activated protein kinases (MAPKs) and the
18 PI3K/Akt/p70 S6 kinase pathway [19-21, 23] and to inhibit the activity of protein
19 tyrosine phosphatases [22]. Further, the zinc-induced metal regulatory transcription
20 factor 1 (MTF1) phosphorylation and target gene expression has been reported to be
21 mediated by PKC, PI3K and JNK dependent pathways [21].

22 The purpose of our current study was to gain a better understanding of the
23 mechanisms of zinc-induced intestinal iron absorption. We have investigated the
24 regulatory role of zinc-sensitive signal transduction pathways and have examined
25 potential interactions with IRPs to regulate iron transporter expression. We

1 hypothesize that zinc-induced signalling events mediate an increase in DMT1
2 expression either directly or via IRP dependent mechanisms.

3

4 **Materials and Methods:**

5 **Materials:** IRP1, IRP2, Akt (P-Ser473 and pan-Akt) antibodies and LY294002 were
6 procured from Cell Signalling Technologies (MA, USA). DMT1 antibody was purchased
7 from Santa Cruz biotechnology (CA, USA). β -actin antibody was from Abcam
8 (Cambridge, MA, USA). The cell culture media components such as antibiotic-mycotic
9 mix and trypsin are procured from Invitrogen (CA, USA). All other reagents were
10 procured from Sigma Chemical Co. (Bangalore, India), unless specified.

11 **Methods:**

12 **Caco-2 cell culture:** The human intestinal Caco-2 cell line was obtained from the
13 American Type Culture Collection (HTB-37, ATCC, Rockville, MD, USA). Caco-2 cells
14 were grown at 37°C in an atmosphere of 5 % CO₂ and 95 % humidity in Eagle's
15 Minimum Essential Medium (MEM) supplemented with 10 % (v/v) heat inactivated
16 foetal bovine serum (FBS), 1 % (v/v) penicillin/streptomycin (Invitrogen, Paisley, UK).
17 For experiments, cells were seeded into 6-well plates and grown for 21 days to allow
18 cells to fully differentiate. The cells were incubated in serum-free MEM for 12h and
19 treated with ZnSO₄ (100 μ mol/L) for the times indicated. LY294002 (25 μ mol/L), where
20 present, was added 30 min prior to the addition of zinc.

21 **Iron uptake:** The measurement of iron uptake by Caco-2 cells has been described
22 previously [16]. Briefly, following zinc treatment, media was removed and replaced
23 with 2mL of 2-(N-morpholino) ethane sulphonic acid (MES)-buffered salt solution (pH
24 6.5 containing: 140 mmol/L NaCl; 5 mmol/L KCl; 1 mmol/L Na₂HPO₄; 1 mmol/L CaCl₂;
25 0.5 mmol/L MgCl₂; 5 mmol/L glucose). Uptake was initiated by the addition of 10

1 $\mu\text{mol/L Fe}^{2+}$ complexed with 1 mmol/L ascorbic acid (freshly prepared prior to the start
2 of each experiment) and 37 kBq/mL $^{59}\text{FeCl}_3$. The reaction was terminated after 15 min,
3 and cell monolayers were washed 3 times in ice-cold transport buffer containing a 10-
4 fold excess of iron to remove non-specifically bound iron, solubilised overnight in 200
5 mM NaOH. The cell associated ^{59}Fe radioactivity was determined by counting in an Auto
6 Gamma Counter (Wizard-2, Perkin Elmer).

7 **Realtime PCR:** Total RNA was isolated from cultured cells using TRIzol. Following cDNA
8 synthesis, expression levels of DMT1 (+IRE and -IRE), IRP2 and β -2 microglobulin
9 mRNA (used as a housekeeping gene) were analysed by real-time quantitative PCR
10 using an ABI Prism 7500 FAST Sequence Detection System and a Power SYBR Green
11 PCR master mix kit (New England Biosciences, UK). The primer sequences used for each
12 gene are given in **Supplementary Table 1**. Quantitative measurements of target genes
13 relative to the housekeeping gene were derived using the ΔCt method. Data are
14 normalised to the untreated control group in each experiment and are presented as the
15 mean \pm S.E.M.

16 **Isolation of polysomes:** IRP-1 and IRP-2 levels, after the incubations, were assessed in
17 the polysomal fraction following treatments as described previously [24]. Briefly, cells
18 were washed in ice-cold PBS and scraped into 3 mL of digitonin buffer (20 mmol/L
19 Tris-Cl, pH 7.4; 250 mmol/L sucrose; 0.007% digitonin; 1 \times protease inhibitor cocktail).
20 Cells were manually homogenized using 21 G and 26½ G needles and kept on ice for 15
21 min. The homogenate was subjected to sequential centrifugation at 1500 g (10 min),
22 10,000 g (10 min) and finally at 100,000g for 60 min. The pellets from the latter two
23 steps enriched in polysomes were pooled and suspended in TX-100 buffer (20 mmol/L
24 Tris-Cl, pH 7.4; 250 mmol/L sucrose; 1% TX-100; 5% protease inhibitor cocktail). The
25 IRP levels in polysomal fraction were assessed by immunoblotting as described below.

1 **Immunoblotting:** Following treatments, the cell monolayers were washed (3X) with 10
2 mmol/L phosphate buffer saline pH 7.2 and lysed in RIPA buffer (Thermo Fisher)
3 supplemented with protease inhibitor cocktail (1X), EDTA (1 mmol/L), Sodium
4 orthovanadate (1 mmol/L), NaF (10 mmol/L). The protein content was estimated using
5 micro-BCA kit method. Equal amount of protein (20-30 µg) was fractionated on 10%
6 SDS-gels under reducing conditions and transblotted on to the PVDF membranes. The
7 blots were blocked with 5% non-fat dry milk or BSA and probed with primary
8 respective primary antibodies followed by respective commercially available secondary
9 antibodies. The blots visualized using enhanced chemiluminescence detection kit (Bio-
10 Rad, USA) and Hyperfilm ECL (Amersham Pharmacia Biotech) or images were acquired
11 using G-box imaging system (Syngene, USA). The blots were re-probed with β-actin,
12 used as a loading control. The images were quantified using Image-J software (NIH,
13 USA) and normalized to respective loading controls.

14 **Transient transfection of Caco-2 cells with siRNA:** Caco-2 cells were seeded at a
15 density of 1.0×10^5 cells/mL in complete media in 12-well plates and allowed to adhere
16 for 10 days. The spent media was aspirated, and the cells were washed once with pre-
17 warmed Dulbecco's Phosphate Buffered Saline (DPBS). Next, the cells were
18 supplemented with OptiMEM (Life Technologies, Paisley, UK) containing 5% FBS
19 without any antibiotics. One hour following the addition of OptiMEM, the Caco-2 cells
20 were transfected with 10 nM of either DMT-siRNA (SLC11A2; M-007381; Dharmacon,
21 CO, USA), IRP2-siRNA (S7498; Life Technologies, Paisley, UK) or a non-targeting
22 scrambled siRNA (AM4635; Life Technologies, UK), using Lipofectamine 3000 (Life
23 Technologies, Paisley, UK) according to manufacturer's protocol. 48 hours after
24 transfection, the media was aspirated from each well, the cells were washed once with

1 pre-warmed DPBS, and supplemented with fresh OptiMEM containing 5% FBS for 72
2 hours.

3 **mRNA stability:** The Caco-2 cells were incubated either in the presence or absence of
4 zinc (100 $\mu\text{mol/L}$) for a period of 4h, followed by addition of actinomycin-D (10 $\mu\text{g/mL}$).
5 At 0, 2, and 4 h after addition of actinomycin D, cells were harvested; qPCR analysis of
6 DMT1 was performed as described above. The mRNA remaining is expressed as a
7 percentage of mRNA levels at $t = 0$ h.

8 **Statistics:** All data are expressed as mean \pm SEM. Statistical analysis was carried out
9 using SigmaPlot (version 12, Systat Software Inc. IL, USA). Student's unpaired t-test was
10 used to compare differences between control and a single test group. One-way or Two-
11 way ANOVA followed by Tukey's post-hoc test was used where appropriate to detect
12 statistical differences ($P < 0.05$) between multiple groups.

13

14 **Results:**

15 Our previous experiments demonstrated that zinc induces iron absorption in
16 differentiated Caco-2 cells with a maximal effect at 100 $\mu\text{mol/L}$ [16], hence all the
17 experiments were performed with this zinc concentration.

18 Initial transport studies with Caco-2 cells grown on Transwell membranes
19 showed a 30% increase in transepithelial iron transport following zinc treatment; this
20 was blocked by the incubation with LY294002 (control, $100.0 \pm 4.8\%$; + zinc, $128.3 \pm$
21 5.7% ; zinc + LY294002, $93.4 \pm 11.5\%$; $P < 0.03$). To determine whether zinc was
22 modulating iron transport at either the apical, or basal, or both surfaces of Caco-2 cells
23 we measured DMT1 and Fpn1 expression. Zinc treatment for either 4 or 24 h
24 significantly induced DMT1 (+IRE) mRNA ($P < 0.01$) and DMT1 protein ($P < 0.001$)
25 expression (**Fig. 1 A and 1B**). DMT1 (-IRE) mRNA levels were low and in some cases

1 below the detection limits of our assay in both control and zinc-treated cells. Thus, we
2 did not measure expression of this isoform. There was no significant effect on Fpn1
3 mRNA in these studies (control, $100.0 \pm 16.0\%$; + zinc 4 h, $108.2 \pm 12.0\%$; + zinc 24 h,
4 $122.1 \pm 19.7\%$). Consistent with the zinc-induced increase in DMT1 (+IRE) expression,
5 zinc treatment also significantly increased ($P<0.001$) iron uptake by Caco-2 cells at the
6 same time points (**Fig. 1C**).

7 To confirm that the effects of zinc on iron uptake were mediated through DMT1
8 we performed siRNA knockdown of DMT1. There was no significant difference in the
9 values obtained for DMT1 protein and iron uptake between the un-transfected control
10 group and cells transfected with scrambled siRNA (**Supplementary Fig. 1**). We
11 therefore used the un-transfected control group for subsequent analysis. DMT1 protein
12 levels were increased in un-transfected cells following exposure to zinc. Treatment with
13 DMT1 siRNA led to significant down regulation ($P<0.001$) of DMT1 protein expression
14 compared to control cells and levels remained significantly suppressed ($P<0.001$) in
15 DMT1 siRNA cells treated with zinc (**Fig. 2A**). DMT1 silencing also significantly
16 inhibited ($P<0.001$) the basal and zinc-induced iron uptake compared to controls (**Fig.**
17 **2B**).

18 Preliminary experiments revealed that zinc-induced iron uptake is inhibited
19 by PI3K but not by JNK or PKC inhibitors (**Supplementary Fig. 2**). Zinc treatment
20 increased phosphorylation of Akt (pSer-473) in a time-dependent manner without
21 changes in total Akt protein expression, and this was blocked completely by LY294002,
22 a potent inhibitor of PI3K (**Fig. 3A**). This prompted us to investigate the role of the PI3K
23 pathway in more detail. Pre-treatment of Caco-2 cells with LY294002 significantly
24 inhibited ($P<0.01$), zinc-induced iron uptake, DMT1 protein and mRNA expression (**Fig**

1 **3B, C and D**). Interestingly, LY294002 treatment alone also significantly inhibited
2 ($p<0.001$) the DMT1 mRNA expression (**Fig. 3D**).

3 Incubation with zinc significantly increased ($P<0.001$) IRP2 protein expression
4 in a time-dependent manner reaching maximum abundance between 0-2 h, and levels
5 remained elevated in the presence of zinc thereafter. However, zinc had no effect on
6 IRP1 expression (**Fig 4A**). Zinc concurrently induced IRP2 levels, but not IRP1, in
7 polysomal fractions (this represents active IRPs bound to IREs in target mRNAs), as a
8 function of time (**Fig 4B**). Zinc did not affect IRP2 mRNA levels over the time course of
9 this study (**Fig 4C**). The effect of zinc on IRP2 protein expression was significantly
10 inhibited ($P<0.001$) by LY294002 (**Fig. 4D**).

11 To determine whether zinc-induced changes in IRP2 expression mediated the
12 regulation of DMT1 we carried out siRNA knockdown of IRP2. There was no significant
13 difference in IRP2 protein levels between the un-transfected control group and cells
14 transfected with scrambled siRNA (**Fig 5A lane 1 and 5**). Transfection with IRP2
15 siRNA, resulted in significant down regulation ($P<0.001$) of IRP2 protein expression
16 compared to un-transfected control cells (**Fig. 5A and B**). IRP2 silencing also
17 significantly reduced ($P<0.001$) the basal DMT1 protein (**Fig. 5A and C**), but not DMT1
18 mRNA expression (**Fig. 5D**) compared to control cells. Furthermore, zinc failed to
19 induce DMT1 protein (**Fig 5A and C**) or mRNA expression (**Fig.5D**) in IRP2-silenced
20 cells compared to control. LY294002 further inhibited ($P<0.01$) the IRP2 and DMT1
21 expression in IRP2-silenced cells (**Fig 5A, B and C**), either in the presence or absence of
22 zinc compared to respective controls.

23 To assess whether the zinc-IRP2 axis increased DMT1 (+IRE) mRNA stability we
24 treated cells with the transcription inhibitor actinomycin D in the presence or absence
25 of zinc. DMT1 (+IRE) mRNA decreased with time; however, the rate of decrease in

1 DMT1 mRNA levels was significantly lower ($P<0.01$) in cells treated with zinc +
2 actinomycin-D compared to cells treated with actinomycin-D alone (**Fig. 6**).

3

4 **Discussion:**

5 Epidemiological studies have shown an association between zinc status, blood
6 haemoglobin levels and iron status. A possible explanation lies in the observation that
7 zinc increases intestinal iron absorption and DMT1 expression in a time-dependent
8 manner [15, 16]. DMT1 is expressed in multiple isoforms that are differentiated
9 primarily by the presence (+) or absence (-) of IRE at the 3'end of the transcribed mRNA
10 [8]. In the present study we determined the expression of these isoforms. The
11 expression of the DMT1 -IRE was below the limit of detection in our assay, whereas
12 DMT1 (+IRE) was significantly increased by zinc.

13 Our previous work has shown that DMT1 is highly expressed at the apical
14 membrane in Caco-2 cells. Using a neutralizing antibody to the transporter we
15 demonstrated that it the primary transporter of non-haem iron in this intestinal cell line
16 [25]. Further, knockout studies in mice demonstrate that DMT1 is essential for
17 intestinal iron absorption [26]. Other putative transporters for iron are present in
18 intestinal cells. For instance, multiple zinc transport proteins (Zips) have been reported
19 to mediate iron uptake in cell culture models [27, 28], and some of these proteins are
20 expressed in Caco-2 cells [29, 30]. However, the physiological relevance of these
21 transporters in mediating iron absorption is unknown. Therefore, to confirm the
22 specificity of DMT1 for iron transport in Caco-2 cells, we studied the impact of DMT1
23 silencing on zinc-induced iron uptake. Silencing of DMT1 led to significant inhibition of
24 iron absorption in Caco-2 cells. These results are consistent with previous data from our
25 group and from others and confirm that DMT1 is the predominant transporter of iron in

1 intestinal cells. Taken together with our mRNA and protein data, these findings indicate
2 that zinc-induced increase iron absorption requires up regulation of DMT1 expression.

3 It is known that cellular iron homeostasis is primarily achieved by post-
4 transcriptional regulation of iron metabolism proteins by IRPs [14, 13]. Binding of IRPs
5 to IREs in 3'-UTR, which are present in DMT1 and TfR1 mRNA, stabilizes the target
6 mRNAs, manifesting in higher expression of protein and thus increased iron absorption
7 [13]. In contrast, enterocyte-specific ablation of IRPs (both 1 and 2) results in
8 malabsorption of iron, leading to death in first four weeks [31]. Mice in these studies
9 exhibited a marked reduction in duodenal DMT1 protein and DMT1 (+IRE) mRNA
10 expression [31]. In the present study, DMT1 (+IRE) mRNA was maximally increased by
11 zinc at 4 h and remained elevated until 24h while the protein expression continued to
12 increase between 4-24 h, implying the possible involvement of post-transcriptional
13 mechanisms mediated by IRPs. Thus, we studied the impact of zinc on IRP1 and 2
14 expression and activity. Zinc had no effect on IRP1 expression but markedly increased
15 the IPR2 protein levels and its localization within polysomal fractions. Since the
16 polysomal IRP levels represent the active IRE-bound pool [24], this suggests that zinc
17 induces both the IRP2 levels and activity. Moreover, time course studies indicated that
18 induction of IRP2 expression and its polysomal abundance occurs as early as 1 h
19 following exposure to zinc, which is in advance of the observed increases in DMT1
20 mRNA and protein level. Therefore, we hypothesized that zinc-induced IRP2 expression
21 might regulate DMT1 post-transcriptionally.

22 To further delineate the mechanism, we studied the impact of IRP2-silencing on
23 zinc-induced DMT1 expression. IRP2-silencing significantly reduced basal DMT1
24 protein levels but not mRNA expression compared to cells transfected with scrambled
25 siRNA. Interestingly, silencing of IRP2 also blocked the zinc-induced DMT1 protein and

1 (+IRE) mRNA expression, suggesting that activation of a zinc-IRP2 axis is required for
2 stabilization of DMT1 mRNA. Treatment with actinomycin-D (a potent inhibitor of
3 transcription) decreased DMT1 mRNA, this was attenuated significantly by zinc
4 treatment, which further supports the notion that zinc induces DMT1 mRNA stability.
5 The fact that zinc had no effect on IRP1 expression and activity, implies a critical
6 mechanistic role of IRP2 in mediating zinc-induced DMT1 expression. In agreement
7 with these results IRP2, but not IRP1, knockout mice exhibited marked reduction in
8 macrophage TfR1 levels [32]. In these animals, IRP2 was demonstrated to be the
9 predominant physiological regulator of iron homeostasis while IRP1 plays an important
10 role during stress conditions such as increased oxygen tension [32]. In addition,
11 increased TfR1 expression in Hela cells following insulin treatment has also been shown
12 to be mediated by IRP2-dependent mRNA stabilization [33]. These results together
13 suggest that zinc increases DMT1 expression in intestinal cells post-transcriptionally via
14 IRP2-dependent DMT1 mRNA stabilization.

15 Zinc is known to induce multiple signalling events, particularly via the PI3K
16 pathway [19, 21, 22, 34]. For example, zinc enhances gastrointestinal barrier function
17 via activation of the PI3K/Akt signalling cascade in intestinal cells [34]. In this study
18 zinc rapidly activated Akt phosphorylation. Inhibition of PI3K with LY294002 blocked
19 zinc-induced iron absorption. These effects were not seen with inhibitors of the JNK or
20 PKC pathways. While LY294002 can block numerous signalling pathways, it is a highly
21 potent inhibitor of PI3K signalling and the inhibition of zinc-induced Akt
22 phosphorylation and DMT1 expression by LY294002 implies a central role for PI3K in
23 mediating these events. In agreement with the activation of a zinc-PI3K/Akt cascade,
24 LY294002 also inhibited zinc-induced IRP2 protein expression. This is consistent with
25 data from others demonstrating that insulin-induced stabilization of TfR1 mRNA in

1 HeLa cells is also mediated via a PI3K/IRP2-dependent mechanism [33]. In addition,
2 zinc has been shown to have insulin-mimetic effects in activating PI3K/Akt signalling
3 cascade in other cell systems [19]. Taken together these results suggest that a zinc-
4 PI3K-IRP2 axis is essential for mediating DMT1 mRNA stability and promoting protein
5 expression. In addition to inhibiting zinc induced DMT1 expression, LY294002 alone
6 also down-regulated DMT1 (+IRE) mRNA levels. This implies that the PI3K pathway
7 plays a critical role in maintaining basal DMT1 expression and intestinal iron
8 absorption. Thus, targeting the PI3K pathway might serve as a therapeutic route to
9 modulate intestinal iron absorption.

10 In summary, these results demonstrate that zinc stimulates intestinal iron
11 absorption by induction of DMT1 expression via a PI3K/IRP2 dependent mechanism.
12 This is the first demonstration that PI3K pathway is involved in regulating the intestinal
13 iron absorption via modulation of IRP2 and could be potentially exploited to improve
14 iron nutrition and metabolism. Given the likely co-existence of iron and zinc deficiencies
15 in populations subsisting on phytic acid-rich vegetarian diets, consideration should be
16 given to improving the zinc status to augment the efficacy of iron supplementation.

17
18

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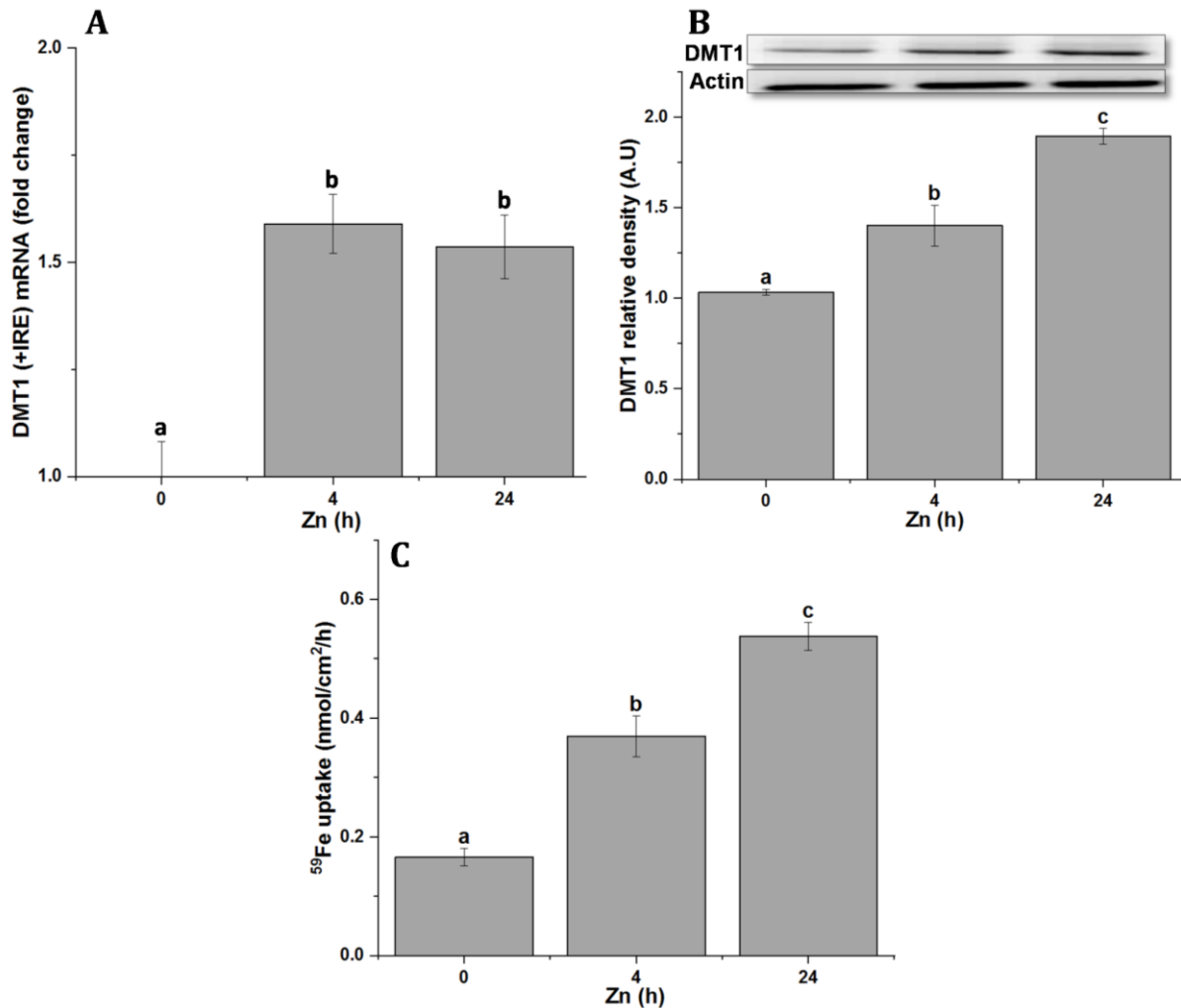
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1 Figure Legends

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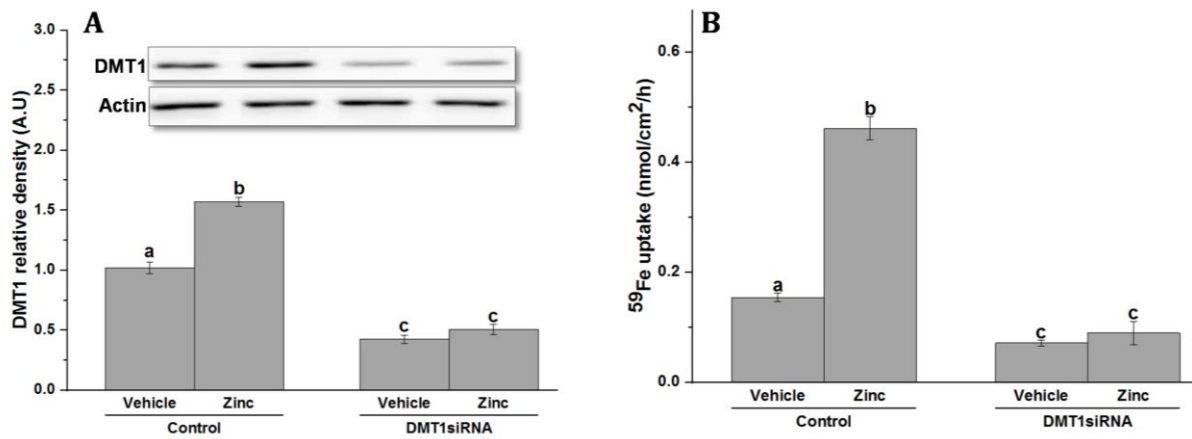
4 **Figure 1. Effect of zinc on iron uptake and DMT1 expression in Caco-2 cells:**
5 Differentiated Caco-2 cells grown in 6-well plates were incubated with Zn (100 μ mol/L)
6 for indicated time. (A) ⁵⁹Fe uptake; (B) DMT (+IRE) mRNA expression; (C) DMT1
7 protein (~65kDa) expression. The iron uptake experiments were performed in
8 triplicate and repeated twice to generate 6 independent observations. The qPCR was
9 performed in triplicate and repeated thrice to generate 9 independent observations, and
10 the data is normalized to the housekeeping gene, the β 2-microglobulin. The
11 immunoblots were repeated thrice, and the same blots were re-probed with β -actin
12 (~45kDa). The densities were normalized to the respective housekeeping gene. The
13 bars indicate the mean \pm SEM and the bars that do not share common superscript differ
14 significantly (P<0.01); Tukey's post-hoc test.

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5 **Figure 2. Effect of DMT1 silencing on zinc induced iron absorption in Caco-2 cells:**

6 Differentiated Caco-2 cells grown in 6-well plates either transfected with DMT1 siRNA
7 (DMT1 siRNA group) or untransfected (control group) were incubated with vehicle
8 (MEM) or Zn (100 μ mol/L) for 24h. (A) DMT-1 protein (\sim 65kDa); (B) ⁵⁹Fe uptake. The
9 iron uptake experiments were performed in triplicate and repeated twice to generate 6
10 independent observations. The immunoblots were repeated thrice, and the same blots
11 were re-probed with β -actin and densities were normalized. Two-way ANOVA found
12 significant interaction between groups (control v DMT1siRNA; $P < 0.01$) and treatment (\pm
13 Zn; $P < 0.01$). The bars indicate the mean \pm SEM and the bars that do not share common
14 superscript differ significantly ($P < 0.001$); Tukey's post-hoc test.

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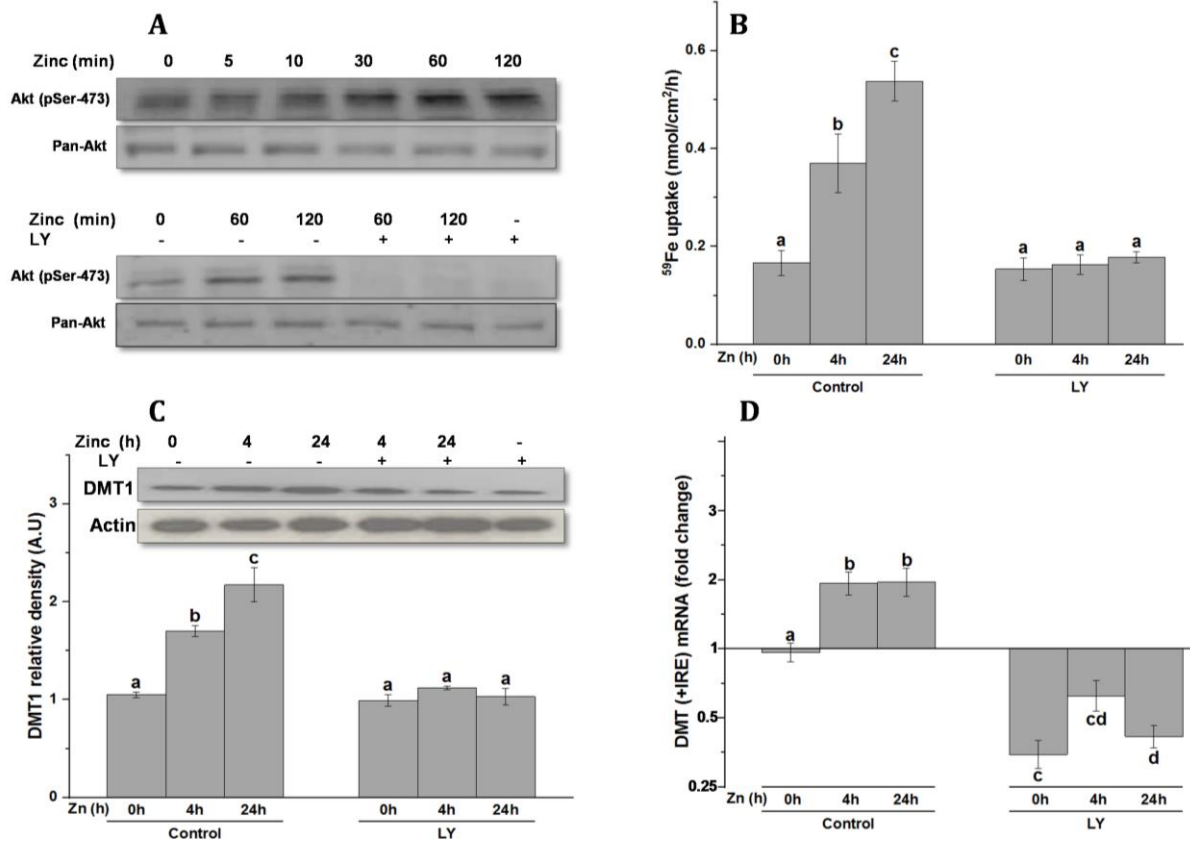
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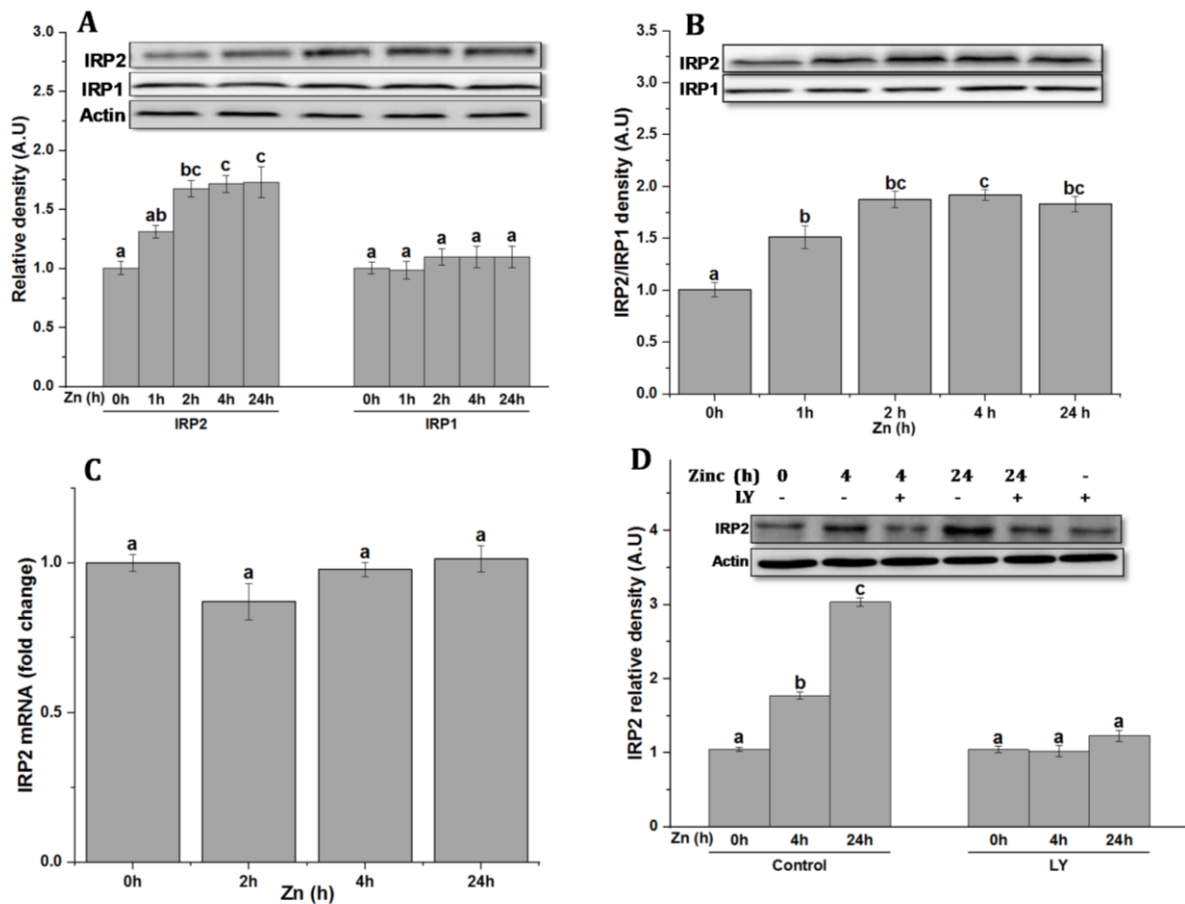
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Figure 3. Effect of PI3K inhibitor on zinc induced iron absorption, DMT1, IRP2 expression: Differentiated Caco-2 cells grown in 6-well plates were incubated with Zn (100 μmol/L) for indicated times either in the absence (control group) or presence of LY 294002 (25 μmol/L; LY group). (A) Time course of Akt (p-Ser473; ~60 kDa) phosphorylation (top panel) and effect of LY294002 on zinc induced Akt phosphorylation (bottom panel) assessed by immunoblotting. (B) ⁵⁹Fe uptake (C) DMT1 (+IRE) mRNA expression (D) DMT1 protein (~65 kDa) expression. The immunoblots were repeated thrice, and the same blots were reported with pan Akt or β-actin as loading controls and the densities are normalized to the respective housekeeping protein. The iron uptake experiments were performed in triplicate and repeated twice to generate 6 independent observations. The qPCR was performed in triplicate and repeated thrice to generate 9 independent observations, and the data is normalized to the housekeeping gene, β2-microglobulin. Two-way ANOVA found significant interaction between groups (control v zinc; P<0.01) and treatment (± LY294002; P<0.01). For B and D there was also a significant interaction between group x treatment (P<0.01). The bars indicate the mean ± SEM and the bars that do not share common superscript differ significantly (P<0.05); Tukey's post-hoc test.

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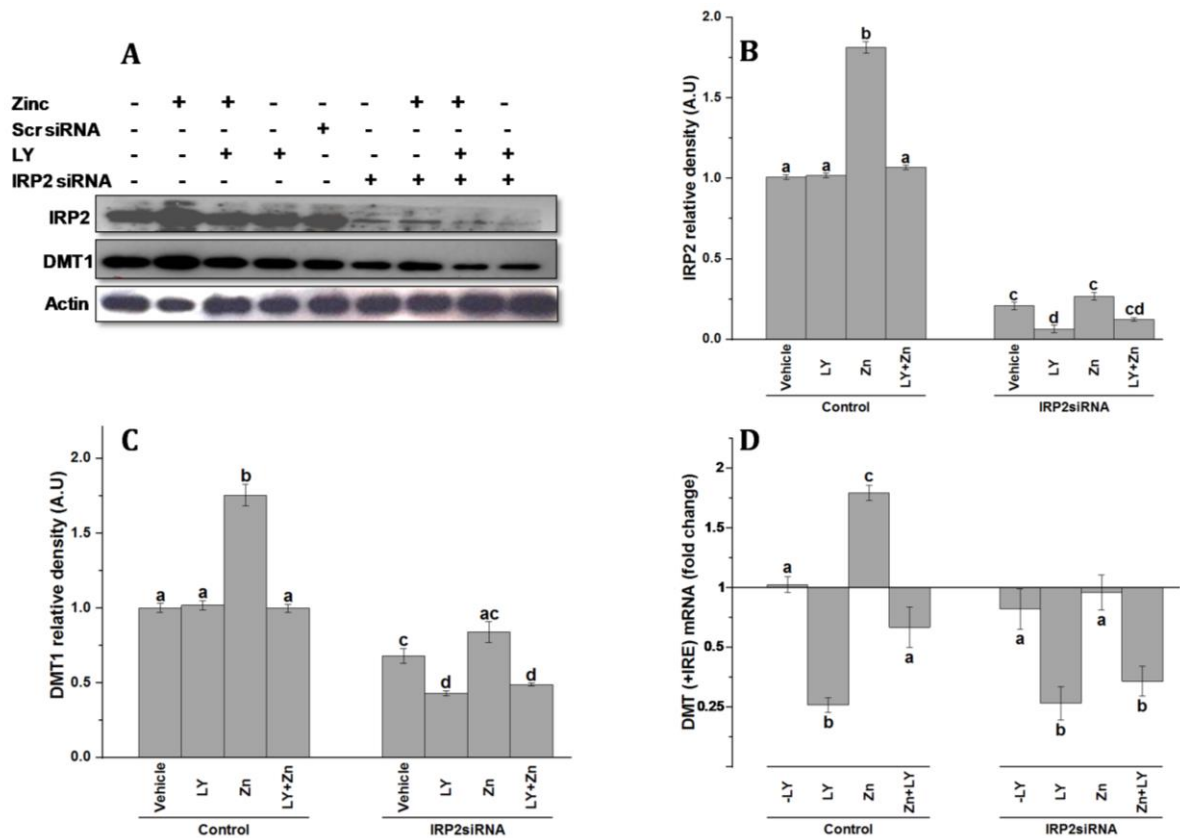
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5 **Figure 4. Effect of zinc and/or PI3K inhibitor on IRP1, IRP2 expression and**
6 **activity:** Differentiated Caco-2 cells grown in 6-well plates were incubated Zn (100
7 $\mu\text{mol/L}$) and/or LY294002 (25 $\mu\text{mol/L}$) for indicated times. (A) immunoblot blot of IRP2
8 (~90kDa) and IRP1 (~90kDa) in total cell lysates (B) immunoblot of IRP2 and IRP1
9 levels in polysomal fraction (C) IRP2 mRNA (D) immunoblot of IRP2 in the presence and
10 absence of zinc and/or LY294002. The qPCR was performed in triplicate and repeated
11 thrice to generate 9 independent observations, and the data is normalized to the
12 housekeeping gene, the β 2-microglobulin. The immunoblots were repeated thrice, and
13 the same blots were re-probed with β -actin. Data were analysed using either one-way
14 ANOVA (A-C) or two-way ANOVA (D). Two-way ANOVA found significant interaction
15 between groups (control v zinc; $P < 0.01$) and treatment (\pm LY294002; $P < 0.01$). There
16 was also a significant interaction between group x treatment ($P < 0.01$). The bars indicate
17 the mean \pm SEM and the bars that do not share common superscript differ significantly
18 ($P < 0.05$); Tukey's post-hoc test.

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3 **Figure 5. Effect of IRP2 siRNA silencing on zinc induced changes in DMT1 protein**
 4 **and mRNA expression:** Differentiated Caco-2 cells grown in 12-well plates were
 5 transfected with IRP2 (IRP2 siRNA group) or control scrambled siRNA or untransfected
 6 (control group) followed by Zn (100 $\mu\text{mol/L}$) and/or LY294002 (25 $\mu\text{mol/L}$) treatment
 7 for 24h. (A) IRP2 (~90kDa) and DMT-1 (~65kDa) immunoblots; (B) densities of IRP2
 8 and (C) DMT1; (D) DMT1 (+IRE) mRNA expression. The immunoblots were repeated
 9 thrice, and the same blots were re-probed with β -actin. The qPCR was performed in
 10 triplicate and repeated thrice to generate 9 independent observations, and the data is
 11 normalized to the housekeeping gene, the β 2-microglobulin. Two-way ANOVA found
 12 significant differences between groups (untransfected v IRP2siRNA; $P < 0.01$) and
 13 treatment (Zn \pm LY294002; $P < 0.01$). There was also a significant interaction between
 14 group x treatment ($P < 0.01$). The bars indicate the mean \pm SEM and the bars that do not
 15 share common superscript differ significantly ($P < 0.05$); Tukey's post-hoc test.

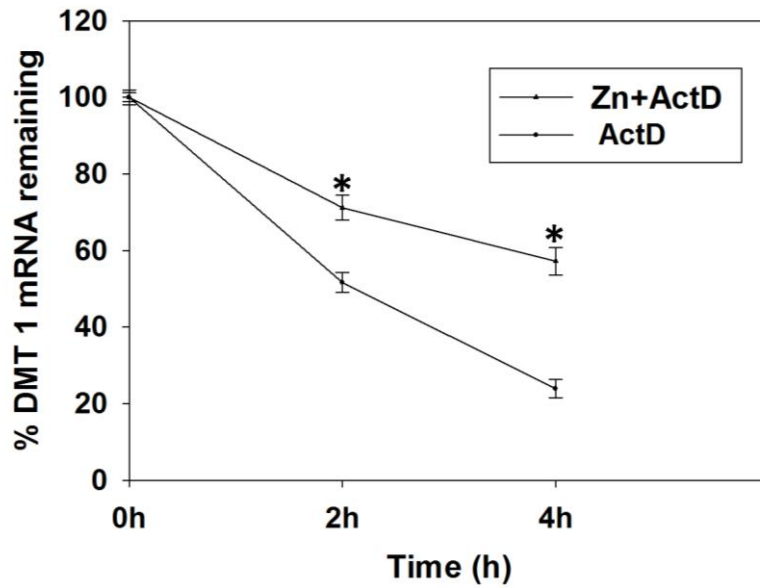
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Figure 6. Effect of zinc on DMT1 mRNA stability: DMT1 mRNA levels in Caco-2 cells incubated either in the presence or absence of Zn and/or actinomycin-D (Act D; 10µg/mL) for 0, 2 and 4h time. The qPCR was performed in triplicate and repeated thrice to generate 9 independent observations, and the data is normalized to the housekeeping gene β 2-microglobulin. Two-way ANOVA found significant interaction between groups (control vs Act D; $P < 0.01$) and treatment (time; $P < 0.01$). * $P < 0.001$ compared to Act D at respective times; Tukey's post-hoc test.