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# Modulation of Dcytb (Cybrd 1) expression and function by iron, dehydroascorbate and Hif-2α in cultured cells

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ABSTRACT

*Background:* Duodenal cytochrome b (Dcytb) is a mammalian plasma ferric reductase enzyme that catalyses the reduction of ferric to ferrous ion in the process of iron absorption. The current study investigates the relationship between Dcytb, iron, dehydroascorbate (DHA) and Hif-2α in cultured cell lines.
*Methods:* Dcytb and Hif-2α protein expression was analysed by Western blot technique while gene regulation was determined by quantitative PCR. Functional analyses were carried out by ferric reductase and $^{59}$Fe uptake assays.
*Results:* Iron and dehydroascorbic acid treatment of cells inhibited Dcytb mRNA and protein expression. Desferrioxamine also enhanced Dcytb mRNA level after cells were treated overnight. Dcytb knockdown in HuTu cells resulted in reduced mRNA expression and lowered reductase activity. Preloading cells with DHA (to enhance intracellular ascorbate levels) did not stimulate reductase activity fully in Dcytb-silenced cells, implying a Dcytb-dependence of ascorbate-mediated ferrireduction. Moreover, Hif-2α knockdown in HuTu cells led to a reduction in reductase activity and iron uptake.
*Conclusions:* Taken together, this study shows the functional regulation of Dcytb reductase activity by DHA and Hif-2α.
*General significance:* Dcytb is a plasma membrane protein that accepts electrons intracellularly from DHA/ascorbic acid for ferrireduction at the apical surface of cultured cells and enterocytes.


## 1. Introduction

The vectorial transport of iron from the lumen of the gut into systemic circulation involves an orchestrated interplay of an apical ferrireductase, luminal and abluminal ferro symporters and a ferroxidase. The components of this transport system-comprising duodenal cytochrome b (Dcytb), divalent metal transporter 1 (DMT1), ferroportin (Fpn) and hephaestin operate in a responsive and regulatory manner for the maintenance of iron homeostasis in mammalian metabolism. This intestinal transit machinery has emerged as a process encompassing transcriptional Hif-2α activation, post-transcriptional IRP-IRE binding and the post-translational regulatory mechanism of hepcidin [7].

Duodenal cytochrome b (Dcytb), also known as cytochrome b reductase1 (Cybrd1), has been suggested as the mammalian plasma membrane protein ferric reductase enzyme that catalyses the reduction of ferric to ferrous ion in the process of iron absorption. Other enzymatic and non-enzymatic mechanisms can render Dcytb activity redundant in some mammalian species. Nevertheless, the need for the function of this reductase may be increased in physiological situations of increased iron absorption particularly [3] in ascorbate auxotrophs such as humans.

Dcytb encodes a putative di-haem protein and shares 40–50% homology with cytochrome b561, an enzyme involved in the regeneration of ascorbic acid (AA) from semi dehydroascorbic acid (DHA) or ascorbate free radical (AFR) [4]. Comparison of the amino acid sequences of Dcytb and cytochrome b561 revealed that the predicted binding motifs for ascorbic acid and DHA present in cytochrome b561 are highly conserved in Dcytb [19]. Dcytb mRNA and protein expressions are induced by modulators for iron absorption despite the fact that Dcytb transcript lacks a definable iron-responsive element. However, the transcriptional modulation of its expression has recently been shown to be regulated by Hif-2α [18,25]. Consequently, activators of Hif-2α could serve as rate-determining factors coordinating the expression of Dcytb, DMT1, and Fpn [18,25]. Specifically, prolyl hydroxylases (PHDs), enzymes that modulate Hif-2α ubiquitination, are reported to be both Fe (II) and $O_2$-dependent and to have maximal activity in the presence of ascorbate; thus, iron deficiency, hypoxia and low ascorbic acid levels

*Abbreviations:* Dcytb, Duodenal cytochrome b; DHA, Dehydroascorbic acid; AA, Ascorbic acid; DFO, Desferrioxamine; Fe-NTA, Ferric-nitrilotriacetate; FAC, Ferric ammonium citrate; HRE, Hypoxia response element; PHDs, Prolyl hydroxylases; VHL, Von–Hippel–Lindau; ARNT, Aryl hydrocarbon receptor nuclear translocator

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[8] enhance Hif-2α levels and culminate in increased Dcytb expression. Paradoxically however, ascorbic acid has been implicated as a substrate for Dcytb in the generation of reducing equivalents for ferric iron reduction [13]. As evidence for Dcytb-ascorbate dependency is lacking, gene silencing was employed in this paper to investigate this requirement in HuTu, a duodenal cell line, because of its relative efficient transient gene transfection efficiency. The current study therefore seeks to elucidate the involvement of Hif-2α, iron and DHA in the expression and function of Dcytb in this line of human intestinal cells.

## 2. Materials and methods

Chemicals and reagents were obtained from Sigma-Aldrich or Merck (Dorset, UK) or otherwise stated accordingly.

### 2.1. Cells and culture conditions

Duodenal HuTu and MDCK cells were obtained from the American Type Culture Collection. Cells were cultured in DMEM medium supplemented with 10% foetal calf serum and with 100 kU/L of penicillin, and 100 mg/L streptomycin. Cells were maintained at 37 °C in a humidified incubator containing 95% air/5% $CO_2$. Cells were trypsinised, plated in 12- or 24-well plates, and grown for 24 h to full confluency for the experiments.

### 2.2. Transfection of cells

Human Dcytb gene fragment was excised from the original vector pMEL18S-FL/Dcytb (Gen Bank accession number AK027115) with EcoR1 and Xho1 and subcloned into a pcDNA 3.1 myc/his(1) mammalian expression vector (Invitrogen). Cells were grown to 60–70% confluency and then transiently transfected with plasmid DNA using Effectene (a non-lipid formulation, Qiagen) according to the manufacturer's protocol. Control cells were transfected with empty pcDNA 3.1 vector. Dcytb-expressing cells were selected with G418 and individual colonies were picked and cultured for the experiments.

HuTu cells were seeded into 12-well-plates to give about 50% confluency 24 h after plating. Cells were transfected with 10 nM of either the siCONTROL non-targeting, non-homologous siRNA (4390843) or siRNA-targeting EPAS1, Hif-2α (S4698), or Dcytb (s230761) (Ambion universal library, Inc., Lafayette, CO, USA) using Lipofectamine RNAi Max (Invitrogen, Carlsbad, CA, USA) according to manufacturer's protocol. Transfected cells were assayed after 72 h for Dcytb and Hif-2α mRNA expression by quantitative RT-PCR, and for ferric reductase and iron uptake assays. Ferric ammonium citrate (FAC), Ferric chloride, $CoCl_2$ and DHA were applied to cells at concentrations and duration that are specified in the legends. Fe-NTA (1:2) has been optimised and used as the substrate for ferric reduction in previous studies [13,19,23,28]. Moreover, the concentrations of FAC used in the current study have been shown to lead to a dose dependent intracellular iron deposition in cells [31,36].

### 2.3. Real time-PCR

Total RNA was extracted from tissue samples using TRIZOL reagent (Invitrogen, U.K.) according to manufacturer's instructions. Quantitative RT-PCR was carried out using an ABI Prism 7000 detection system in a two-step protocol with Roche Universal primers and probes. Quantitative measurement of each gene was normalised to the threshold cycle value for 18S ribosomal RNA.

Sequences of human primers used, forward and reverse respectively are as follows:

Dcytb F 5′-aactggcacccagtgctc-3′
R 5′-cggcagtctgtagacgatga-3′

Hif-2α F 5′-gacatgaagttcacctactgtgatg-3′
R 5′ gcgcatggtagaattcatagg-3′

18S F 5′-gcaattattccccatgaacg-3′
R 5′-gggacttaatcaacgcaagc-3′

### 2.4. Ferric reductase and iron uptake assays

Ferric-nitrilotriacetate (Fe-NTA) reduction was determined on HuTu cells as described previously [13,37] measuring the absorbance of ferrozine-chelable Fe(II) at 562 nm. Ferric reductase and uptake assays were performed on cells after the various experimental treatments. Iron uptake was assayed in Hank's Balanced Salt Solution buffer (pH 7.0) and freshly prepared $^{59}Fe^{(III)}$-NTA (1:2) in a final concentration of 10 μmol/L iron. Uptake was initiated by the addition of 250 μL of assay buffer to the cells and terminated after 60 min unless otherwise stated. Cells were washed 3 times in ice-cold Versene (0.2 g EDTA/L PBS) to remove non-specifically bound $^{59}Fe$. Cells were collected in 200 μL of 2% sodium deoxycholate in 10 mmol/L Tris and radioactivity measured with a gamma counter (LKB Wallace 1280). Thereafter 20 μL of the cell extract was used for protein concentration determination according to Bio-Rad assay protocol (Bio-Rad Laboratories, UK).

### 2.5. Western blot analysis

The duodenal mucosa samples were scraped with a glass slide and homogenised (in a buffer containing 50 mM mannitol, 2 mM HEPES, 0·5 mM PMSF and pH 7·2) with an Ultra Turrax (IKA, Staufen, Germany) homogeniser in (3 × 30 s pulses on full speed). The homogenate was centrifuged at 1500*g* for 5 min and the supernatant was centrifuged for 1 h at 15,000*g* to obtain the crude membrane fraction. Nuclear extract was prepared using NaCl high salt buffer [1]. Protein concentration was determined using Bio-Rad reagents (Bio-Rad Laboratories, USA). Fifty (50) μg of membrane protein extracts were loaded on a 12% gel in a SDS-PAGE. The proteins separated were then transferred to Hybond ECL-nitrocellulose membrane (Amersham Biosciences, Bucks, UK) using a Bio-Rad semi dry transfer apparatus (Bio-Rad Trans-Blot$^R$ SD Semi-Dry Transfer Cell). Membrane was blocked with 5% milk for 1 h and probed with Dcytb polyclonal, HIF-2α (Novus Biologicals USA) β-tubulin (Abcam, UK) or β-actin (Sigma, UK) antibodies diluted in 0.01% milk in TBS. Cross-reactivity was observed with peroxidase-linked anti-IgG by using SuperSignal West Pico (Thermo Scientific, USA).

### 2.6. Statistical analysis

All values are expressed as means ± SE from at least 3 different experiments. Statistical differences between means were calculated using Student's *t* test in correcting for differences in sample variance. When multiple comparisons were necessary, 1-way or 2-way ANOVA was performed, using SPSS 14 with Tukey's post hoc test. Differences were considered significant at $P < 0.05$.

## 3. Results

### 3.1. Modulation of Dcytb expression by iron, desferrioxamine (DFO) or DHA.

A dose response downregulation of Dcytb protein expression by ferric ammonium citrate (FAC) was shown in MDCK cells (Figs. 1A and B). Treating stably transfected MDCK Dcytb expressing cells with dehydroascorbate (DHA) led to a down regulation of Dcytb protein levels (Figs. 1C and D). More importantly, treatment of HuTu cells with iron (as $FeCl_3$) reduced Dcytb mRNA expression. The effect of DHA on Dcytb mRNA expression was slightly inhibitory after an 18-h exposure while that of DFO was enhancing (Fig. 1E).

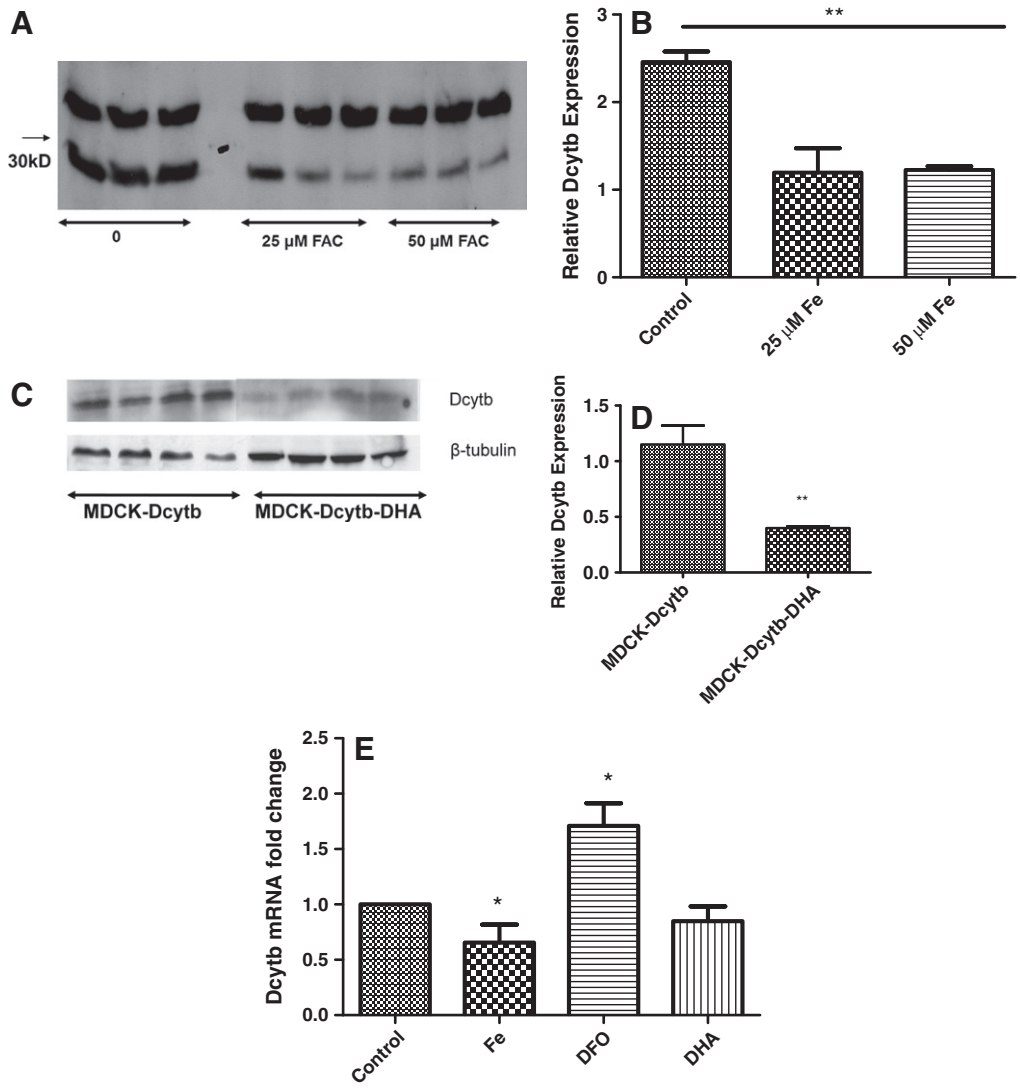



**Fig. 1.** Modulation of Dcytb expression in cultured cells by iron (as ferric ammonium citrate, FAC, or $FeCl_3$), desferrioxamine (DFO) or dehydroascorbate (DHA). (A) Dcytb protein expression by cells treated with 25 or 50 μM of Fe (as FAC). Cells were exposed to FAC overnight, and membrane protein fractions were subjected to Western blot analysis. Values are means ± SE of three experiments ($P < 0.05$). (B) Densitometric plot of protein bands in cells treated overnight with FAC. (C) Dcytb protein expression in cells treated with 200 μM DHA for 24 h. (D) Densitometric plot of protein bands in cells treated with 200 μM DHA for 24 h. (E) Dcytb mRNA expression of HuTu cells that were treated with 100 μM Fe (as $FeCl_3$), 200 μM DHA, or 50 μM DFO. Expression levels were quantified by real time PCR.

### *3.2. Hypoxia induction with $CoCl_2$ enhanced Hif-2α protein expression, ferric reductase activity and iron uptake in HuTu cells*

HuTu cells treated for 18 h with 500 μM $CoCl_2$ revealed enhanced expression of Hif-2α protein levels (Fig. 2A). This was associated with increased ferric reductase activity (Fig. 2B) and a modest increase in iron uptake (Fig. 2C).

### *3.3. Dcytb gene silencing, reductase activity and iron uptake in HuTu cells*

HuTu cells that were transiently transfected with siRNA Dcytb duplex oligos exhibited about 80% reduction ($P < 0.01$) in Dcytb mRNA expression when compared with the siCONTROL non-targeting, non-homologous siRNA (Fig. 3A). Furthermore, Fe-NTA reductase activity was also significantly reduced in cells transfected with Dcytb siRNA (Fig. 3B), though to a lesser degree ($P < 0{\cdot}05$). However, only moderate inhibition was achieved with $^{59}$Fe-uptake (Fig. 3C). DHA enhanced ferric reductase activity in HuTu cells. DHA pre-treatment for 4 h resulted in higher ferric reductase activity than 72 h incubation with DHA (Fig. 3D). Pre-treating HuTu cells with 200 μM $FeCl_3$ or 500 μM DHA led to reduced iron uptake (Fig. 3E). Moreover, pre-treating Hutu cells with Dcytb antisera for 1 h exerted an inhibitory modulating effect on reductase activity in HuTu cells (Fig. 3F). Furthermore, the current study employed gene silencing to explore the ascorbate-dependent ferric reductase activity of Dcytb. HuTu cells that were transiently transfected with Dcytb siRNA duplex or siCONTROL siRNA were assayed after 72 h. Impermeant ascorbate oxidase (1 U/mL) was added to the medium to degrade extracellular AA and scavenge any AA leaking from the DHA-pretreated cells. Cells were preloaded with 50 μM or 100 μM DHA for 4 h, before ferric reductase assay was performed for 1 h in the presence of ascorbate oxidase (1 U/mL). As expected, DHA significantly enhanced ferric reductase activity in control HuTu cells (Fig. 3G) but not in Dcytb-silenced cells. This finding implies that Dcytb exerts a DHA-dependent ferric reducing functionality in these cells.

### *3.4. Hif-2α gene silencing, reductase activity and iron uptake in HuTu cells*

HuTu cells that were transiently transfected with siRNA Hif-2α duplex oligos showed an approximate 50% reduction in Hif-2α mRNA

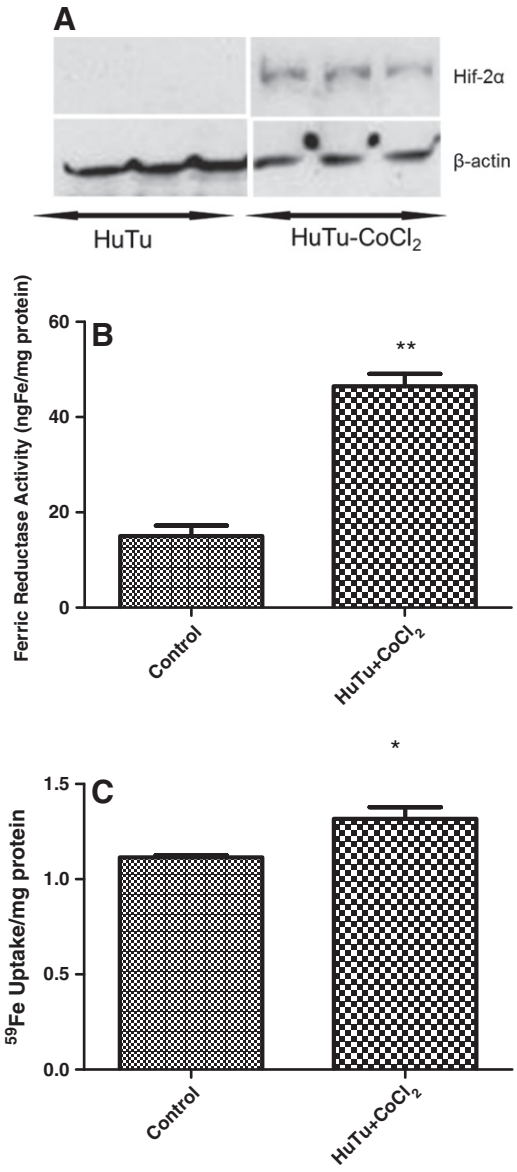



**Fig. 2.** Hypoxia induced with $CoCl_2$ enhanced Hif-2α protein expression, ferric reductase activity and iron uptake in HuTu cells. (A) Hif-2α protein expression in HuTu cells exposed for 18 h to chemically-induced hypoxia with 500 μM of $CoCl_2$. (B) Ferric reductase activity for 1 h in HuTu cells pretreated overnight to chemical hypoxia with 500 μM of $CoCl_2$. (C) $^{59}Fe$ uptake in HuTu cells after 18 h exposure to chemical hypoxia with 500 μM of $CoCl_2$. Values are means ± SE of three experiments ($P < 0.05$).

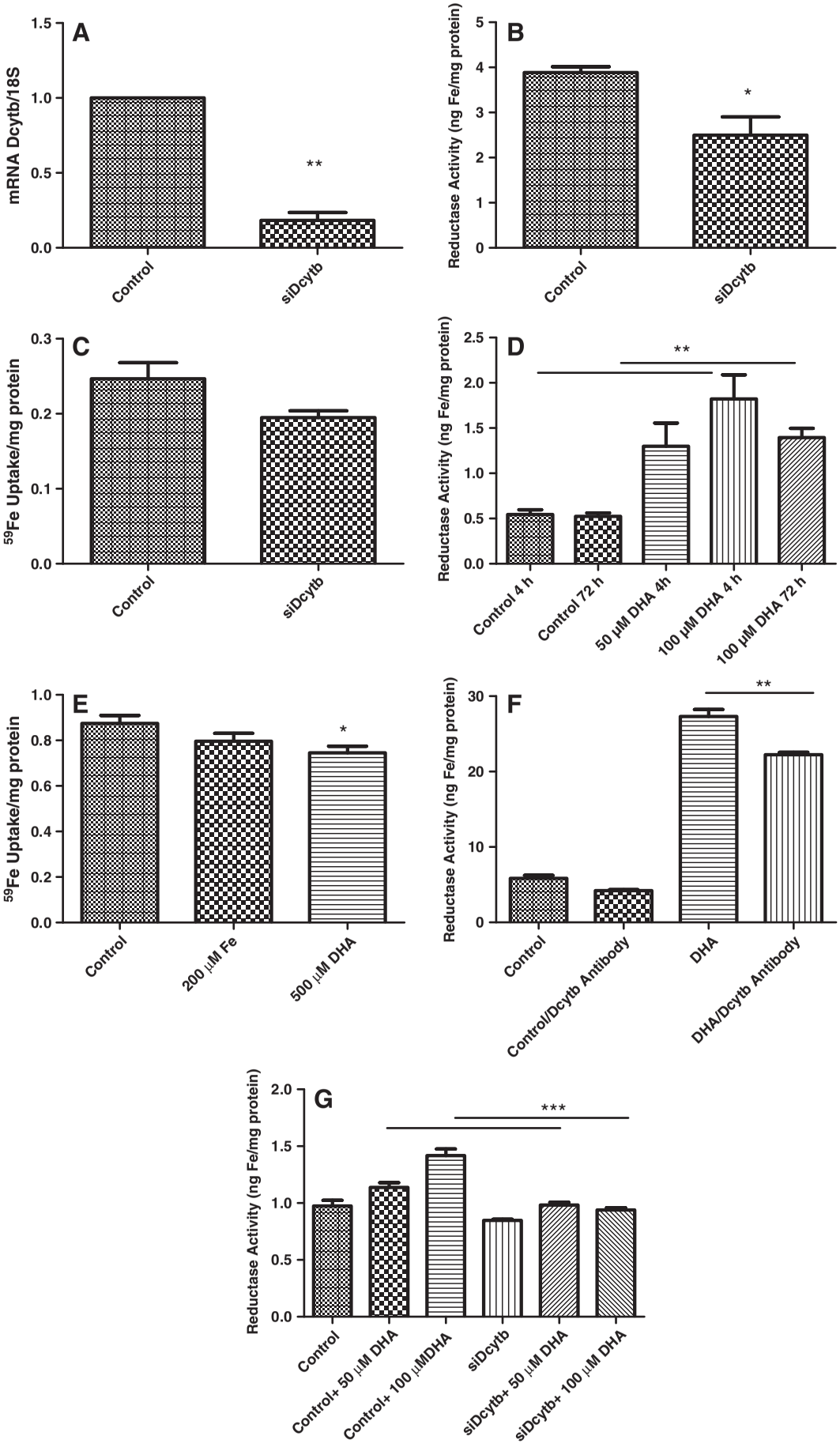
expression when compared with siCONTROL (non-targeting, non-homologous siRNA (Fig. 4A). However, Hif-2α mRNA silencing, resulted only in about 25% inhibition of Fe-NTA reductase activity in these cells (Fig. 4B). This finding is consistent with the levels of reduction obtained for $^{59}Fe$-NTA uptake in Hif-2α gene silenced HuTu cells (Fig. 4C).

## 4. Discussion

Several ferric reductases have thus far been identified and characterised in mammalian iron metabolism. However, plasma membrane ferrireductase, Dcytb, remains the principal enzyme localised in duodenal enterocytes of the gastro-intestinal tract [20]. Dcytb is regulated in situations of iron deficiency [19], hypoxia [15] and haemolytic anaemia [5] in mice. Consistent with this observation is the modulation of Dcytb protein expression by iron and DFO in cultured cells in the current study (Fig. 1e). Given the absence of the canonical IRE elements that typify some other iron metabolism genes, iron-dependent regulation of Dcytb has until recently been a conundrum. Moreover, only one functional isoform or transcript of the gene has been identified and characterised. Compelling evidence from two independent studies reported the orchestration of the transcriptional regulation of Dcytb by Hif-2α in mice [18,25]. HIF-2α is one of 3 members of Hif transcriptional factors that are responsive to hypoxia or oxygen status in cellular metabolism. The cascade of Hif regulation commences with the oxygen-dependent hydroxylation by PHDs of prolyl residues on HIF-2α, a component of HIF heterodimer. This creates a binding epitope for Von–Hippel–Lindau (VHL) which, upon binding HIF-2α, triggers ubiquitination or degradation of the subunit of the complex. This degradation is enhanced by iron or ascorbate. Thus, Dcytb mRNA and protein expression are decreased by the presence of iron or DHA (Fig. 1). While HIF-2α exerts a transcriptional regulation of Dcytb mRNA levels, the mechanisms by which Fe and DHA influence protein levels are not clear. Both endogenous and transgenic Dcytb expressions are also modulated post-transcriptionally (Supplementary Data). The mechanism by which Hif-2α mediates the regulation of transgenic Dcytb cytomegalovirus (CMV) promoter is not clear. However, it is reasonable to suggest that this mediation might be achieved either through interaction with other sites on the Dcytb cDNA transcript or, alternatively, through a post-transcriptional mechanism that remains to be determined. Conversely, hypoxia (reduced $O_2$ or chemically induced by $CoCl_2$), or reduced ascorbate levels can stabilise HIF-2α for binding to Hif-1β or Aryl hydrocarbon receptor nuclear translocator (ARNT) which together constitute the HIF transcription complex. The resultant complex binds to hypoxia response elements (HREs) to induce transcription of Dcytb gene. Chemical induction of hypoxia by $CoCl_2$ enhanced ferric reductase activity and iron uptake in HuTu cells (Fig. 2). This finding agrees with previous observations *in vivo* in both hypobaric [26,27] or $CoCl_2$ induced hypoxia in several studies in mice.

While a loss-of-function phenotype in Dcytb knockout mice might be confounded *in vivo* by extenuating variables [6], the primary ferrireductase function of Dcytb in transgenic cultured cells is incontrovertible. This is apparent in Dcytb gene-silenced cells employed in the current study. Dcytb gene knock-down resulted in decreased ferric reductase activity in HuTu cells (Fig. 3). Although its specific and selective ferric reducing activity could in reality be substituted or competed for by the presence of other reductases and luminal non-enzymatic reducing agents, Dcytb expression is responsive to increased physiological demands in systemic metabolism [3]. While there are reports of DMT1-independent non-haem iron uptake in brain cells [11,31,38], the phenotypic manifestation of anaemia in DMT1 KO mice accentuates ferrous iron as the dominant species that is absorbed particularly in duodenal enterocytes. DMT1, could however, in hepatocyte be redundant [35].

Consistent with the function of Hif-2α in the regulation of Dcytb and DMT1 expression in mice [18,25], gene silencing led to significant inhibition of ferric iron reduction and iron uptake in HuTu cells in the current study. Hif-2α silencing also reduced ferritin synthesis by the same magnitude in HuTu cells exposed to Fe-NTA (unpublished). Hif-2α knock-down resulted in direct functional consequences on Hif target genes that are associated with ferrireduction and iron uptake (Fig. 4). Low transfection efficiency of siRNA oligos in particular might have accounted for lower magnitude of inhibition of iron uptake in Hif-2α silenced cells. Moreover, the enzymatic function of Dcytb as a ferrireductase seems more plausible than that of constituting a downstream effector of iron absorption. Ascorbate has been shown to stimulate ferricyanide reduction through a mechanism different from the classical NADH-dependent plasma membrane reductase [11,12]. Human erythrocytes exhibit a transmembrane electron transfer activity that uses intracellular ascorbate as a source of electrons to reduce extracellular ferric ions [32]. Dcytb has been localised on the membrane of human erythrocytes and is implicated in the maintenance of iron in the ferrous state [29]. Likewise, Caco-2 and MDCK cells heterologously expressing


A
mRNA Dcytb/18S
1.5
1.0
0.5
0.0
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Control
siDcytb
B
Reductase Activity (ng Fe/mg protein)
5
4
3
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1
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Control
siDcytb
C
59Fe Uptake/mg protein
0.3
0.2
0.1
0.0
Control
siDcytb
D
Reductase Activity (ng Fe/mg protein)
2.5
2.0
1.5
1.0
0.5
0.0
**
Control 4 h
Control 72 h
50 μM DHA 4h
100 μM DHA 4 h
100 μM DHA 72 h
E
59Fe Uptake/mg protein
1.0
0.8
0.6
0.4
0.2
0.0
*
Control
200 μM Fe
500 μM DHA
F
Reductase Activity (ng Fe/mg protein)
30
20
10
0
**
Control
Control/Dcytb Antibody
DHA
DHA/Dcytb Antibody
G
Reductase Activity (ng Fe/mg protein)
2.0
1.5
1.0
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Control
Control+ 50 μM DHA
Control+ 100 μMDHA
siDcytb
siDcytb+ 50 μM DHA
siDcytb+ 100 μM DHA

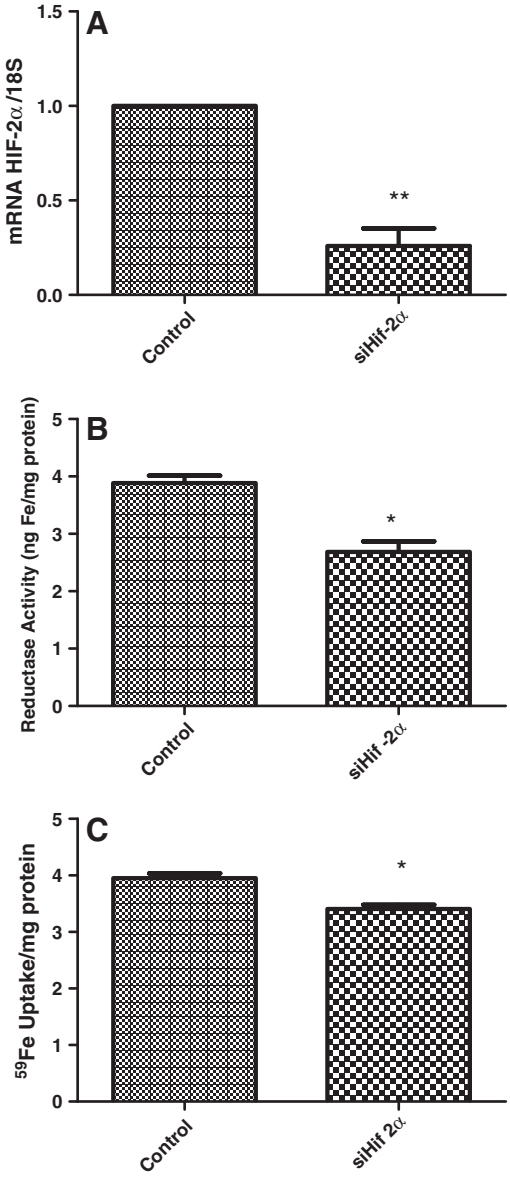


**Fig. 4.** Hif-2α gene silencing, reductase activity and iron uptake in HuTu cells. (A) Silencing of Hif-2α gene by siRNA, 10 nM of either the siCONTROL non-targeting, non-homologous siRNA or siRNA targeting Hif-2α was transfected into HuTu cells and RNA was extracted after 48 h. Values are means ± SE of three experiments ($P < 0.05$). (B) Ferric reductase activity assayed 72 h after Hif-2α gene silencing in HuTu cells (C) $^{59}$Fe uptake 72 h after Hif-2α gene silencing in HuTu cells. Uptake of $^{59}$Fe, 10 μmol/L Fe-NTA was assayed for 1 h. Values are means ± SE of three experiments ($P < 0.05$).

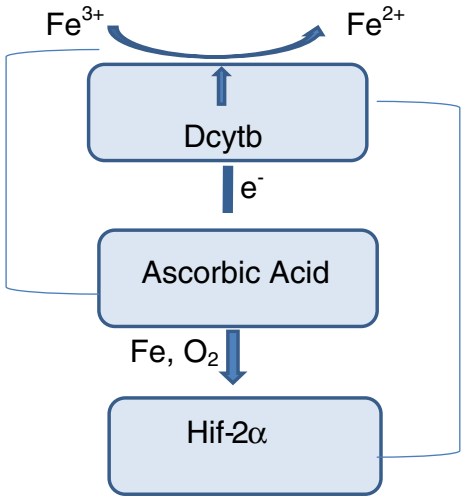


**Fig. 5.** Functions of DHA/ascorbic acid in ferrireduction in cellular metabolism. Ascorbic acid that is generated intracellularly from reduced ascorbate, dehyroascorbate (DHA), provides electrons for extracellular ferrireduction by Dcytb. Moreover, non-enzymatic ferrireduction by ascorbic acid could also occur extracellularly on the apical membranes of cells. Conversely, ascorbic acid levels increase prolyl hydroxylase activity in the presence of iron (Fe) in normoxia to induce ubiquitination of Hif-2α thereby inhibiting Dcytb expression.

Dcytb demonstrated enhanced ferric reduction of Fe-NTA when preloaded with DHA [13,37]. Pre-incubation of HuTu cells with a high concentration of DHA exerted a reduction in iron uptake (Fig. 3e). This result extends the observation reported by Scheers et al. [24] in Caco-2 cells. A 4 h pre-incubation of HuTu cells with 100 μM DHA, however, enhanced iron uptake (unpublished data). Gene silencing was used in the current study to uncouple the ascorbate-dependent Dcytb ferric reductase activity in HuTu cells. Dcytb expression levels in HuTu cells regulated the magnitude of ferric reduction irrespective of the amount of DHA preloaded onto the cells. Ferric reduction in the presence of the substrates was therefore limited by low levels of Dcytb reductase enzyme. This was also evident with DHA dependent Dcytb antisera inhibition of reductase activity (Fig. 3f). Employing oxidised DHA instead of ascorbic acid, coupled with the presence of extracellular impermeable ascorbate oxidase, ensured that intracellular ascorbate served as the sole source of reducing equivalents for the extracellular reduction of ferric iron. Consequently, Dcytb possibly catalyses electron transfer from intracellular ascorbate directly to extracellular ferric or cupric ions [22,37] or ascorbate from the cytosol directly reduces extracellular ferric ions [11]. Alignment of the transmembrane topology of Dcytb with that of cytochrome b561 has shown that the ascorbic acid binding motif of both proteins is located intracellularly while those of the ascorbyl free radical (AFR) are extracellular. The putative membrane configuration of Dcytb is consistent with the orientation of plasma ascorbate/AFR oxidoreduction [10,30]. Recombinant expression of Dcytb in insect Sf9 cells [22] and in *Escherichia coli* [16,17] revealed four critical histidine residues that might putatively bind the two haem moieties in the molecule. Moreover the midpoint reduction potentials of the haem residues in Dcytb were shown to be reducible by ascorbate in a manner analogous to those of cytochrome b561.

The high basal ferric reductase activity could be due to other electron reducing equivalents donors such as endogenous ascorbate [14,33,34], glutathione [21] or quercetin [33]. Consequently, degeneracy of ferrireduction under some conditions confers redundancy to Dcytb in iron metabolism. Moreover, high background flux levels obscure marked

**Fig. 3.** Dcytb gene silencing, reductase activity and iron uptake in HuTu cells. (A) Silencing of Dcytb gene by siRNA, 10 nM of either the siCONTROL non-targeting, non-homologous siRNA or siRNA targeting Dcytb was transfected into HuTu cells and RNA was extracted after 48 h. Values are means ± SE of three experiments ($P < 0.01$). (B) Ferric reductase activity was assayed 72 h after Dcytb gene silencing in HuTu cells. Values are means ± SE of three experiments ($P < 0.05$). (C) $^{59}$Fe uptake (as $^{59}$Ferric-nitrilotriacetate, Fe-NTA) in HuTu cells 72 h after Dcytb gene silencing in HuTu cells. Uptake of $^{59}$Fe, 10 mmol/L Fe-NTA was assayed for 1 h. Values are means ± SE of three experiments ($P < 0.05$). (D) DHA enhanced ferric reductase activity in HuTu cells. Cells were pretreated with dehydroascorbate (DHA) at 50 μM or 100 μM for 4 or 72 h after which ferric reductase activity was carried out in a buffer containing ascorbate oxidase (1 U/mL). (E) HuTu cells that were pre-treated with 200 μM of iron as ferric ammonium citrate (FAC) or 500 μM of dehydroascorbate (DHA) overnight after which $^{59}$Fe uptake (as $^{59}$Ferric-nitrilotriacetate, Fe-NTA) was carried out for 1 h. (F) HuTu cells that were pre-treated with Dcytb antibody for 1 h. Following this, ferric reductase activity was carried out for 1 h. (G) HuTu cells were transfected with siCONTROL or siDcytb oligos and pre-treated with 50 or 100 μM dehydroascorbate (DHA) for 4 h prior to determination of ferric reductase activity. Ferric reductase activity was determined for 1 h in a buffer containing ascorbate oxidase (1 U/mL) to degrade extracellular ascorbate generated during the assay. Different lines above data bars indicate that these groups are significantly different ($P < 0.05$).

differences that might be attributed to alterations in Dcytb. It is pertinent that the existence of a plasma membrane AA/DHA cycling that promotes non-transferrin-bound iron (NTBI) reduction and uptake by human erythroleukemia (K562) cells has been reported [9] as constituting cellular influx of DHA, which is reduced to AA by cytosolic reducing equivalents. Consequently, AA is shuttled extracellularly for ferric iron reduction and uptake into the cell. The results of our study form a basis for further studies on pH measurements of Dcytb-dependent transplasma reductase-coupled proton transport. Ascorbic acid is consequently dualistic in its function in ferrireduction in mammalian iron metabolism. It is, on the one hand, a catalyst required to activate prolyl hydroxylases that are involved in ubiquitination of Hif proteins in normoxia which consequently down-regulate Dcytb expression [18,25]. This is particularly relevant when DHA or ascorbic is applied to cells for a long duration [24] Incidentally, this role may also be made redundant by the presence of glutathione [21] intracellularly. In contrast, ascorbic acid could also directly reduce ferric to ferrous ion in luminal and systemic metabolism, in addition to serving as a source of electrons [2] for Dcytb reductase activity (Fig. 5).

In conclusion, Dcytb is an iron-regulated ferric reductase that is transcriptionally regulated by Hif-2α. Consequently, gene silencing of Hif-2α culminated in reduced ferric reductase activity and iron uptake in HuTu cells. Furthermore, plasma membrane Dcytb is possibly a conduit that accepts electrons generated intracellularly from ascorbic acid for ferrireduction at the apical surface of cultured cells and enterocytes.

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