Moonlighting cell surface GAPDH recruits Apo Transferrin to effect iron egress from mammalian cells

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Abstract
Iron homeostasis is a tightly regulated process with precise control of its influx and egress from cells. Though mechanisms of its import into cells via iron carrier molecules are well characterized, iron export remains poorly understood. The current paradigm envisages unique functions associated with specialized macromolecules for its cellular import (transferrin receptors) or export (ferroportin). Earlier studies have revealed that iron-depleted cells recruit Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a moonlighting protein to their surface for internalization of the iron carrier holo transferrin (holo Tf). Here we report that under the contrary condition of intracellular iron excess, cells switch the isoform of GAPDH on their surface which now recruits iron-free apo transferrin in close association with ferroportin to facilitate efflux of iron. Increased surface GAPDH expression synchronized with increased apo Tf binding and enhanced iron export from cells, a capability lost in GAPDH knockdown cells. These findings were confirmed in vivo utilizing a rodent model of iron overload. Besides identifying for the first time an apo transferrin receptor, our work uncovers two-way switching of multifunctional molecules for managing cellular micronutrient requirements.

Introduction
Though iron is an essential micronutrient for all life it is a double edged sword. It constitutes an essential component of various proteins crucial for oxygen transport and electron transfer. At the same time excess of iron catalyses the formation of highly reactive free radicals (Fenton’s reaction), which damage bio-molecules through peroxidation (Dunn et al., 2007; Ganz, 2007; Hentze et al., 2004; Knutson and Wessling-Resnick, 2003). Accumulation of iron in cells and tissues causes medical complications, including cirrhosis, liver cancer, pancreatic failure, cardiomyopathy and arthritis (Burke et al., 2001; Kong et al., 2008). Iron overload also affects the central nervous system and has been implicated in pathogenesis of Parkinson’s and Alzheimer’s disease (Fleming and Ponka, 2012; Kong et al., 2008). It is therefore imperative for organisms to constantly maintain a control of iron metabolism at all the different steps involving iron turnover. This involves tight regulation of; absorption (enterocytes), usage (erythroid cells), recycling (reticuloendothelial cells) and iron storage (macrophages and hepatocytes) (Fleming and Ponka, 2012).

Practically all of extracellular iron in blood is chelated to transferrin, an abundant serum iron transport protein. Under physiological conditions Tf saturation is maintained ~30% leaving no excess free iron available to cause toxicity (Sheftel et al., 2012). Though the cellular iron...
uptake pathways via Tf-Transferrin receptors 1 & 2 (TfR1 also known as CD71 and TfR2) are well characterized, knowledge regarding iron export is still limited (Ganz, 2007). Ferroportin, also known as iron regulated transporter 1 (IREG1), Metal transporter protein 1 (MTP1) or Slc40a1, is the only known iron exporter in mammalian cells (Abboud and Haile, 2000; Donovan et al., 2000; McKie et al., 2000). It is expressed in duodenal enterocytes, tissue macrophages and hepatocytes where it participates in cellular release of iron (Abboud and Haile, 2000; Canonne-Hergaux et al., 2006; Donovan et al., 2000; McKie et al., 2000; Ramey et al., 2010). Iron loading of cells enhances the localization of ferroportin to plasma membrane of macrophages (Delaby et al., 2005), however the exact mechanism by which it transports iron and how the exported iron is sequestered remains unclear (Ganz, 2007; Le Gac et al., 2013; Wessling-Resnick, 2006).

Previously it has been shown that the multifunctional glycolytic enzyme Glyceradehyde-3-phosphate dehydrogenase functions as receptor for Holo transferrin (Holo Tf) & Lactoferrin (Lf) on the surface of a diverse range of cell types including macrophages and upon iron starvation many cells prefer to use surface localized GAPDH for Tf-iron uptake rather than TfR1 (Kumar et al., 2012; Modun et al., 1998; Modun et al., 2000; Raje et al., 2007; Rawat et al., 2012). Recently we have also demonstrated that cells enhance their secretion of GAPDH into the extracellular milieu (sGAPDH) when they are depleted of iron. This sGAPDH functions as an autocrine / paracrine receptor which traffics Tf-iron into various tissues and cell types (Sheokand et al., 2013). In the current study we report an additional dimension to this multifunctionality of GAPDH where the same protein demonstrates contrasting behavior under the opposing condition of cellular iron status (i.e. excess intracellular iron) to maintain iron homeostasis. Using cell types that play a key role in maintaining iron homeostasis (cells of reticuloendothelial system, hepatocytes and enterocytes) we provide evidence that, when exposed to iron overload, cells again enhance their recruitment of GAPDH to the membrane surface. However this GAPDH is an alternate isoform that differs from the GAPDH recruited upon iron starvation. It does not bind Holo Tf, instead it interacts with Apo Tf with high affinity and facilitates the export of iron from cells. This capability was lost in cells where GAPDH knockdown was performed. We further demonstrate that in iron loaded cells GAPDH interacts with ferroportin on the surface of cells which may facilitate the loading of iron exported by ferroportin on to Apo Tf. Results from cell culture experiments were validated in vivo utilizing rodent models of iron overload.

Results
Modulation of cell surface GAPDH upon iron overload: Earlier we have demonstrated that iron starvation enhances surface GAPDH expression and many cells prefer this portal for Tf-iron acquisition instead of the well studied transferrin receptor 1 (Kumar et al., 2012). We were interested to investigate the response of this receptor system upon iron excess in cells. Towards this end we first confirmed cellular iron loading by two independent methods. Cells that play a key role in iron homeostasis were monitored for increased intracellular iron levels upon incubation with iron. A gradual increase in intracellular iron peaking at 12 hours was observed (Fig. S1A). In addition iron loading of macrophages was also achieved by erythrophagocytosis (EPG) a phenomena that occurs naturally in organisms during clearance and recycling of effete erythrocytes (Fig. S1B & inset S1B).

Surface TfR1 expression decreased in iron loaded cells (Fig. S1C & D) which is consistent with earlier reports (Auriac et al., 2010; Casey et al., 1988) of cells trying to limit further iron import. At the same time, we detected an increased surface expression of GAPDH on primary macrophages, hepatocytes and macrophage cell lines along with a decrease in case of primary enterocytes (Table I). In vivo experiments gave similar results. Peritoneal macrophages harvested from mice that had been injected i.p. with iron dextran or opsonized RBC’s also demonstrated enhanced surface GAPDH (Table I). Increase in surface GAPDH was time and dose dependent in respect to iron loading and did not involve any change in intracellular GAPDH levels (Fig. 1A &B). Earlier we had reported an increase in GAPDH on the surface of iron starved cells (Kumar et al., 2012; Raje et al., 2007). When compared with those observations, we noticed that the extent of increase in GAPDH expression on membrane was significantly higher upon iron loading of cells as compared to iron depletion (Fig. 1C).

GAPDH expressed on cell membrane of iron overloaded cells is of a different form than that observed upon iron starvation: To determine if there was any difference in the nature of GAPDH expressed on cell surface upon iron overload as compared to iron depletion we looked at the membrane partitioning of GAPDH in the two cases. Our previous work had demonstrated an increase in the FCDR ratio [ratio of flow cytometric signal of target molecule in detergent resistant membrane fraction (DRM) versus total signal(Gombos et al., 2004)] of GAPDH upon iron starvation of cells indicating that the GAPDH presented on the cell surface upon cellular iron depletion is preferentially localized to the detergent resistant fraction of membrane (Kumar et al., 2012). Interestingly, in case of iron overloaded cells, though surface GAPDH increased to a greater extent, no significant change in the FCDR ratio, as compared to control cells, could be observed (Fig. 1D&E), suggesting that the
increased GAPDH is equally distributed between both membrane fractions. Analysis of membrane protein fractions by 2-D gel electrophoresis and western blotting revealed an alkaline shift in the predominant GAPDH isoforms of iron loaded cells as compared to iron depleted cells (Fig. 1F). LC-MS/MS analysis of membrane GAPDH from iron depleted cells revealed a higher abundance of numerous post translational modifications (PTMs) including oxidation, dimethylation, acetylation, nitrosylation and phosphorylation as compared to GAPDH from membrane of iron loaded cells (Fig. 2A-C).

The increase in surface GAPDH upon iron overload also did not result in any increase in the cellular capacity to bind Holo Tf as reported earlier for iron depleted cells (Kumar et al., 2012), in fact there is a significant decrease (Fig. 3A). These results collectively demonstrate that GAPDH recruited to cell surface during iron overload is a different isoform than that presented upon iron starvation.

Modulation of Apo Tf binding upon iron overload: As excess iron is deleterious, iron loaded cells would seek to divest themselves of this excess of metal. Apo Tf, abundantly present in serum, is among the best known biological chelators of iron and we checked for its recruitment by iron loaded cells. Macrophages and hepatocytes demonstrated an increased binding of Apo transferrin which correlated with the increase in surface GAPDH expression (Fig. 1A-D, Fig. 3B and Table I). In case of primary enterocytes surface GAPDH decreased along with a corresponding decrease in Apo Tf binding (Table I). GAPDH knockdown THP1 cells which fail to enhance surface GAPDH upon iron overload also lacked Apo Tf binding (Table I). The increase in Apo Tf binding by cells also matched the time dependent increase in surface GAPDH upon iron loading (Fig. 3C and 1A).

Apo Tf binding to cells: Equilibrium dissociation constant (K_D) of Apo Tf binding to surface of excess iron treated J774 cells was calculated to be 1.11 nM (Fig. 3D) suggesting the presence of a high affinity receptor. This is similar to the value of 1nM reported for TfR1 and there could be some concern that the affinity reported in this case is due to Tf binding to CD71 either in apo form or after conversion into holo Tf. However TfR1 does not bind Apo Tf at physiological pH (Andrews, 2000) and Apo Tf was prevented from conversion to the holo form by inclusion of DFO in the incubation buffer(Kawabata et al., 2000). FACS analysis demonstrated that Apo Tf binding to cells is inhibited in presence of excess of unlabeled ligand (Fig. 3E) indicating that it is a specific process. In addition the binding was decreased when cells were pretreated with proteolytic enzyme pronase (Fig. 3F) confirming that the receptor involves a protein molecule (Kumar et al., 2012).
**GAPDH and Apo Tf interact in vitro:** As surface GAPDH appeared to be a receptor for Apo Tf binding on iron loaded cells we decided to first check if GAPDH and Apo Tf interact in vitro. To evaluate this, plate based solid phase interaction and SPR assays were performed. GAPDH coated in ELISA wells specifically captured Apo Tf (Fig. S2A). Biacore assay revealed an equilibrium dissociation constant (K_D) of 5.3 nM for the GAPDH-Apo Tf interaction (Fig. S2B).

**GAPDH and Apo Tf co-localize and interact on cell surface:** To ascertain whether this strong in vitro interaction of GAPDH and Apo Tf occurs in vivo, we investigated association and interaction of GAPDH with Apo Tf on surface of iron treated J774 cells. Utilizing confocal microscopy GAPDH was observed to co-localize with Apo Tf on the surface of cells (Fig.4A, Fig. S2C&D). Acceptor photobleaching based Foster resonance energy transfer (FRET) assay also demonstrated interaction between surface GAPDH and Apo Tf (Fig. 4B&C, Fig. S2E). Finally, co-immunoprecipitation of biotinylated Apo Tf with GAPDH from membrane of iron loaded cells confirmed the interaction between the two proteins (Fig. 4D).

**Correlated modulation of surface GAPDH and Apo Tf binding in iron loaded rats:** We utilized an in vivo rat model to confirm whether iron loading indeed increases cell surface GAPDH and Apo Tf binding. Liver and serum iron estimation confirmed the iron overload in a rat model (Fig.S3A&B). Hepatocytes, peritoneal macrophages and enterocytes were examined for surface expression of GAPDH and Apo Tf binding as compared to cells isolated from control animals. While hepatocytes and peritoneal macrophages of iron loaded rats demonstrated an increase in surface GAPDH expression along with a corresponding increase in Apo Tf binding, enterocytes demonstrated a decrease in both surface expression of GAPDH and Apo Tf binding (Fig. 5A & S3C). The change in cell surface expression of GAPDH upon iron loading correlated well with the change in Apo Tf binding by all three cell types studied (Fig 5B).

**Apo Tf recruited by GAPDH facilitates iron export:** To understand the physiological significance of increased Apo-Tf capture by iron loaded cells we explored its role in facilitation of iron export from cells. Having established that incubation with iron supplemented medium causes maximum intracellular iron accumulation within 12 hrs (Fig. S1A), we chose this as a start point to evaluate iron exit. When Apo Tf was included into the incubation medium, iron loaded J774 cells demonstrated a significant enhancement of iron export into extracellular medium within 1 hr, as assayed by the chromogenic iron assay (Fig. S4A). Exit of iron was also quantitated using cells labeled with ^55^Fe. We observed an increase in iron export from various iron loaded (by FeCl₃ incubation or EPG) cells when
incubated with Apo Tf (Fig. 6A & B). J774 and THP1 cells that had not been subjected to any iron loading (untreated control cells) also demonstrate a small baseline change in iron export when Apo Tf was added into the incubation medium (Fig. S4B). Interestingly, incubation with Apo Tf caused a sharp increase in iron export from untreated enterocytes while in iron loaded enterocytes the effect of Apo Tf was minimal (Fig. S4B & Fig. 6A).

Addition of Apo Tf failed to enhance iron export in untreated or iron loaded GAPDH knockdown THP1 cells (Fig. 6A-B & S4B). In addition this effect of Apo Tf in enhancing iron exit was dose dependent and could not be brought about by Holo Tf (Fig. 6C). We further confirmed that iron exported out of cells is sequestered into Apo Tf (Fig. 6D) by precipitating the added Apo Tf, and checking for presence of protein bound radioactive iron.

Surface GAPDH colocalizes and interacts with ferroportin: Ferroportin, the only known iron exporter in mammals, is known to localize to the cell membrane upon iron loading (Delaby et al., 2005). Having observed that in iron loaded cells, surface GAPDH recruits Apo Tf to facilitate cellular iron export (Fig. 4-6) we decided to evaluate the possibility of any GAPDH-ferroportin interaction on surface of these cells. Confocal microscopy demonstrated simultaneous colocalization of all three proteins on cells (Fig. 7A). Acceptor photobleaching based foster resonance energy transfer (FRET) analysis demonstrated the interaction between GAPDH and ferroportin (Fig. 7B-D). Finally, co-immunoprecipitation of surface GAPDH and ferroportin confirmed the interaction between the two proteins (Fig. 7E).

Discussion:
Iron metabolism has long been an area of active research (Ganz, 2013) and while extensive studies have been conducted to characterize the mechanistic basis of iron import by cells the field of cellular iron export is still poorly explored. In our earlier studies, an increase in the recruitment of GAPDH to cell surface which enhanced Tf-iron trafficking, was observed when cells were depleted of iron, demonstrating its role in maintaining essential levels of cellular iron (Kumar et al., 2012; Raje et al., 2007). To fully comprehend its role in iron homeostasis we investigated as to how this molecule responds when cells are loaded with iron (under such situations TfR1 expression on cells is known to be down regulated). Interestingly, we once again observed an increase (to an even greater extent than observed upon iron depletion) in the surface expression of GAPDH of macrophages and hepatocytes. Unlike our earlier observations in iron starved cells (Kumar et al., 2012) and also in agreement with an earlier report which showed that iron loading did not alter GAPDH expression in rat liver (Quail and Yeoh, 1995), no change in intracellular GAPDH levels upon iron loading was observed. These observations suggest that the increase in membrane
GAPDH could be due to redeployment of the existing intracellular protein to the membrane. Since membrane GAPDH accounts for a tiny fraction of the vast pool of intracellular GAPDH (Seidler, 2013), no discernible alteration in cytosolic levels would be perceptible. Our results also demonstrate that the GAPDH recruited to the cell membrane upon iron overload is of a different isoform than that deployed upon iron depletion. Existence of discrete GAPDH isoforms catalyzing independent functional activities are well known (Glaser and Gross, 1995; Seidler, 2013) and a switching of GAPDH in membrane of bone marrow derived macrophages upon iron loading to a more alkaline isoform has been reported recently (Polati et al., 2012). Our LC-MS/MS results demonstrate that GAPDH recruited upon iron loading lacks phosphorylation and acetylation and also has a lower abundance of several PTMs including oxidation, dimethylation and nitrosylation in comparison to GAPDH recruited upon iron depletion. These modifications are well known for their ability to shift the isoelectric point of proteins as observed by us in this study as well as by numerous other workers previously (Choudhary et al., 2000; Grillon et al., 2012; Kuyumcu-Martinez et al., 2007; Madian et al., 2012; Park et al., 1988; Zhang et al., 2011). The presence of numerous PTMs in GAPDH has been well established in earlier reports (Seidler, 2013; Seo et al., 2008).

Unlike the GAPDH recruited upon iron depletion (Kumar et al., 2012), GAPDH expressed on the membrane during iron overload does not bind Holo Tf. This is expected as under these conditions cells are already coping with an excess of the metal and would seek to restrict further iron import via Holo Tf. On the other hand these cells have an urgent need to externalize their excess iron. As high levels of extracellular free iron is deleterious the exported iron needs to be expeditiously chelated from the vicinity of the cell (Hentze et al., 2004). One of the most abundant and high affinity chelators of iron in serum capable of this task is Apo Tf (Sheftel et al., 2012) and the existence of an independent Apo Tf receptor for such a purpose has been speculated (Umbreit, 2005). We examined Apo Tf binding to surface of iron loaded cells and observed an increase upon iron loading. This correlated with the increased surface GAPDH in various cells analyzed. Cells in which GAPDH had been knocked down and do not express GAPDH on their surface either under normal conditions or upon iron overload, completely failed to bind Apo Tf (Table I). The binding of Apo Tf was modulated in a time dependent manner similar to increase in surface GAPDH. In addition it was specific, saturable and pronase sensitive demonstrating the involvement of a protein receptor with an affinity of ~1nM. Earlier investigations have suggested the presence of such specific, saturable and pronase sensitive binding of Apo Tf onto rat peritoneal macrophages involving very high affinity receptors and independent of Holo Tf binding (Nishisato and
Aisen, 1982). However till date no specific molecule has been identified as an Apo Tf receptor. Our data suggests that this high affinity receptor for Apo Tf on the surface of macrophages is an isoform of GAPDH.

Biacore based analysis revealed affinity of GAPDH-Apo Tf interaction to be of the same order as Tf-TfR1 interaction and also to correlate with the binding affinity of Apo Tf with iron loaded cells. Confocal microscopy based co-localization, co-immunoprecipitation and FRET based assays further confirmed their interaction on cells. A study utilizing K562 cells suggested the existence of common Apo and Holo Tf binding sites (Xiu-Lian et al., 2004). However, K562 cells do not express GAPDH on their surface at all (Kumar et al., 2012) and those results may be due to the existence of some alternate binding sites.

Our studies provide evidence that interaction of Apo Tf with iron loaded cells facilitates the exit of iron and this ability is lost upon knockdown of GAPDH. These results demonstrate that surface GAPDH along with Apo Tf are crucial for iron egress. The role of Apo Tf in iron export has been demonstrated previously with mixed results. It enhanced iron release from rat kupffer cells, bone marrow macrophages (Kondo et al., 1988; Rama et al., 1988) and hypoxic macrophages in presence of ceruloplasmin (Sarkar et al., 2003). In vivo studies have demonstrated that iron infused to decrease plasma iron binding capacity (decreased serum Apo Tf) reduces, post EPG cellular iron release (Bergamaschi et al., 1986; Lipschitz et al., 1971; Siegenberg et al., 1990) and rat bone marrow macrophages exhibit a suppressed iron release when incubated with saturated transferrin (Rama et al., 1988). However, other groups have reported that Apo Tf has no effect on iron release after infusion of RBC’s (Lipschitz et al., 1971) or after EPG in isolated rat peritoneal macrophages (Saito et al., 1986).

As ferroportin is a well established iron exporter in mammals, we investigated if surface GAPDH interacts with ferroportin and confirmed this to be so in iron loaded macrophages. We hypothesize that GAPDH recruits Apo Tf onto the cell surface, thereby increasing its local concentration in close proximity to ferroportin, so that iron exported through ferroportin is rapidly chelated. This is critical as localized high concentrations of free iron around the cell membrane can cause lipid peroxidation and compromise membrane integrity. Studies in Xenopus oocytes (which express ferroportin) have demonstrated the requirement of Apo Tf for increased iron efflux (Donovan et al., 2000). The increase in cell surface GAPDH upon iron overload, observed by us, also matches with the previously reported alterations in ferroportin expression in different cell types. Upon iron loading ferroportin expression is decreased on the basolateral surface of enterocytes so as to decrease iron entry into systemic circulation (Thomas and Oates, 2004). On the other hand its expression is increased on the
surface of macrophages and hepatocytes to facilitate iron export out of these cells for maintaining homeostasis (Delaby et al., 2005; Ramey et al., 2010). In accordance with these reports, we have observed a decreased surface GAPDH expression, decrease in Apo Tf binding and a marginal effect on iron export, in iron loaded enterocytes upon incubation with Apo Tf while control untreated enterocytes recorded a significant increase in iron export when incubated with Apo Tf. This can be explained by the fact that enterocytes are involved in dietary iron absorption and they export iron absorbed from gut lumen into systemic circulation, thus making it available to the whole organism. During presence of excess iron, they would seek to minimize their iron export into plasma so as to restrict addition of more iron into circulation. Macrophages and hepatocytes demonstrated increased surface GAPDH and Apo Tf binding upon iron overload (as has been reported for ferroportin) to maintain iron homeostasis. Our in vivo studies in iron loaded rats validated our results from cell culture experiments. We found an increase in surface GAPDH expression and Apo Tf binding in hepatocytes and peritoneal macrophages while both decrease in case of enterocytes as compared to cells isolated from control rats.

GAPDH is a highly multifunctional protein with a diverse range of functions (Sirover, 2011; Sirover, 2012). Previous investigations had revealed an additional role for GAPDH as a receptor for trafficking of holo-transferrin into iron starved cells. Here we demonstrate that by changing the isoform recruited to the membrane, GAPDH additionally functions as a high affinity receptor for Apo-Tf thereby maintaining iron homeostasis by enhancing cellular iron egress.

Our current studies uncover an additional order of multifunctionality for GAPDH (multifunctionality within multifunctionality) wherein it switches its role under the diametrically opposite conditions of cellular iron status providing a two way switch based on modification of PTMs for cellular iron regulation. In addition, to the best of our knowledge this is also the first report which clearly identifies a high affinity Apo Tf receptor and provides a clear mechanism for its role in managing cellular iron. A schematic model of this dual role of GAPDH is outlined by us (Fig 7F).

Materials and Methods

Cell Lines, primary cells and materials: All cell lines (including a stable THP1 cell line in which knockdown of total cellular GAPDH had been previously established), primary cells (Sprague Dawley rat peritoneal and spleen macrophages, enterocytes, hepatocytes) were obtained/purified and maintained essentially as described previously (Sheokand et al., 2013). THP1 cells were activated for 24 hours with 12.5ng/ml of phorbol 12- myristate 13-acetate
All cells were maintained in RPMI-1640 medium supplemented with 10% fetal calf serum.

Purified RBC’s were labeled with CellVue® claret far red fluorescent cell linker midi kit (Sigma) as per manufacturer’s instructions. Rabbit anti RBC serum was raised and validated using standard methods. Erythrocytes were opsonized by incubation with antisera (1:50 dilution) at 37°C for 1 hr followed by 3x wash with PBS. Animal experiments and collection of blood from healthy volunteers was with due approval from relevant institutional ethical committees.

**Cell treatments:** The iron concentration of cells was increased by culturing cells in medium containing 100µM FeCl₃ as described previously (Foster et al., 2001). Excess iron was thus presented as ferrous ascorbate to increase solubility and facilitate cellular accumulation (Han et al., 1995). Iron in macrophage cells was also enhanced by erythrophagocytosis which was performed by incubating a monolayer of macrophages with opsonized RBC’s (50:1) for 24 hrs in complete medium (Knutson et al., 2005). Controls were set up in parallel with normal media. Extracellular RBC’s were lysed with distilled water for 2 minutes and then rinsing cells with neutral buffer (20mM HEPES, 150mM NaCl, 5mM KCl and 1mM each of CaCl₂, MgCl₂). For *in vivo* acute iron loading *Sprague Dawley* rats were injected i.p. 100 mg Fe dextran (Sigma) or 1 x 10⁹ opsonized RBC’s and after 24 hrs, peritoneal macrophages were isolated as described previously (Sheokand et al., 2013). For iron depletion experiments, cells were cultured in complete medium with 100µM Desferrioxamine (DFO) as described previously (Kumar et al., 2012). No significant change in cell viability due to any treatment was observed as assessed by several independent viability assays described earlier (Kumar et al., 2012; Sheokand et al., 2013), in addition a Caspase-Glo™ 3/7 assay kit (Promega) was also utilized to confirm absence of any inducement of cell apoptosis.

**Protein Conjugates:** Rabbit muscle GAPDH was obtained from Sigma while Apo transferrin was procured from Calbiochem. Proteins were conjugated with fluorochromes or biotin by standard procedures using FITC (Sigma), Hilyte Fluor 647 Protein labeling kit (Anaspec) and sulfo-NHS-LC biotin (Pierce).

**Calcein quenching assay:** Erythrophagocytosis was performed with monolayer of macrophages as described above in cell treatments with controls set up in parallel with normal media. Subsequently cells were washed three times with serum free medium (SFM) and incubated with 500 nM Calcein-AM at 37°C for 10 min. After extensive washing with SFM the fluorescence of intracellular dye was measured by FACS. As Calcein fluorescence
is quenched by iron a decrease in cellular fluorescence is indicative of an increased intracellular labile iron pool.

**Enzyme Activity of Cell Surface GAPDH**: Ectoenzyme activity analysis of intact control or FeCl₃ treated J774 cells was performed essentially as described previously (Raje et al., 2007).

**FACS analysis**: All FACS experiments were performed as described previously (Raje et al., 2007). Briefly, 2 x 10⁵ cells/tube were stained with, either 1µg anti-GAPDH antibody (calbiochem) / isotype control (mouse IgG), followed by sheep anti mouse-FITC (Fab)₂ (Sigma) or with anti CD71(TfR1)-PE/ isotype control (BD) or with 10µg HoloTf Alexa 647 / ApoTf Alexa647. For Apo Tf staining, 100 µM DFO was included in incubation buffer so as to prevent conversion of Apo form of Tf to Holo Tf. For intracellular GAPDH staining, cells were fixed with 1% paraformaldehyde and permeabilized using 0.1% saponin at 37°C for 15 min before staining. Analysis of 10⁴ cells was done for each sample using FACS Calibur flow /FACS Verse cytometer (BD).

**Flow cytometric differential detergent resistant ratio (FCDR) of GAPDH**: Control or iron loaded J774 cells were analyzed for FCDR ratio of surface GAPDH exactly as described previously (Kumar et al., 2012).

**2D analysis of membrane GAPDH**: J774 cells were treated with DFO or excess iron and membrane fractions were prepared essentially as described earlier (Raje et al., 2007). Membrane proteins (200 µg from either, DFO or FeCl₃ treated J774 cells) were processed for clean up by Biorad protein clean up kit® as per manufacturers instructions and subsequently dissolved in solubilization buffer (2 M thiourea, 7 M urea, 3% CHAPS, 20 mM Tris) to a final volume of 125 µl. IPG 7 cm, pH 3–10 linear gradient strips (BioRad) were loaded with samples by rehydration-loading. Isoelectric focusing was performed at 0-250 V for 2 h (linear), 250 V for 1 h (rapid), 250 V-3000V for 4 h (linear), 3000V maintained until 15,000 V-hours achieved. The current was limited to 50 µA per strip, and the temperature was kept at 20 °C for all isoelectric focusing steps. For the second dimension SDS-PAGE, the IPG strips were incubated in equilibration buffer 1 (6 M urea, 2% SDS, 20% glycerol, 0.375 M Tris–HCl pH 8.8, 2% DTT) for 10 min followed by incubation in equilibration buffer 2 (6 M urea, 2% SDS, 20% glycerol, 0.375 M Tris–HCl pH 8.8, 2.5% iodoacetamide) for another 10 min and then transferred onto 4%–15% gradient acrylamide gels (Biorad). The gels were run at 25 mA until the bromophenol blue front had reached the bottom of the gel. Resolved proteins were processed for western blotting and immunodetection of GAPDH as described previously (Raje et al., 2007).
LC-MS/MS analysis of membrane GAPDH from iron depleted and iron treated cells: J774 cells were treated with DFO or excess iron and membrane fractions were prepared as described above. Extracted membrane proteins (200 μg from either, DFO or FeCl₃ treated J774 cells) were run on a SDS PAGE and stained with coomassie brilliant blue. Bands corresponding to GAPDH were excised and destained with 200mM of NH₄HCO₃ in 40% acetonitrile (ACN) at 37°C for 30 min. Subsequently gel pieces were dried in a Speed Vac® and incubated at 37°C in digestion buffer containing 20ng/μl of trypsin (sigma), 40mM of NH₄HCO₃ in 9% acetonitrile for 18 hr. Finally, supernatant was colletted for digested peptides and subjected to LC-MS/MS analysis. Peptides were analyzed by ultra high performance liquid chromatography (UPLC)/ESI/MS/MS with mass spectrometer (Q-TOF 6550, Agilent). Peptides were separated using a C₁₇ reversed phase 3.5μm, 2.1x150mm analytical column (X Bridge™ BEH 300, Waters), A sample volume of 8μl was injected and flow rate was maintained at 400μl/min. Mobile phase constituted 90% solution A(0.1% formic acid, 90% water,10% ACN) and 10% of solution B(0.1% formic acid, 90% ACN, 10% water) for 15 mins , 70% solution A : 30% solution B for 8 min, 40% solution A : 60% solution B for 5 min, 10% solution A : 90% solution B for 2 min, 50% solution A : 50% solution B for 2 min and 90% solution A :10% solution B for 2 min over a time period of 32 min. Capillary voltage was 1.5 kV and dry gas flow rate of 13 l/min was used with temperature of 220°C. The scan range used was 100-3200 m/z. The tandem mass spectra were annotated and peak list files (.MGF) were generated. Data analysis was performed essentially as described earlier (Seo et al., 2008). Protein identification was performed by searching in National Center for Biotechnology Information non redundant database (NCBInR) using Mascot program (matrix science) with following parameters: peptide mass tolerance, 1.2Da; MS/MS ion tolerance, 0.6Da; taxonomy was limited to Mus musculus; allow up to 1 missed trypsin cleavage site; variable modifications considered were: acetylation(K), deamidation(N,Q), methylation(D,E), dimethylation(N,R,K), oxidation(M,C), phosphorylation(S,T,Y), cysteine propionamide, Pro->Pyro-Glu(P), Pyro-Glu(N-term, E,Q), nitrosylation(C), succinylation(K), ADP-ribosylation(K), Palmitoylation(C), Myristoylation(N term G), Farnesyltion(C), GPI Anchor (Protein C term). Only significant hits as defined by MASCOT probability analysis were considered. In addition, a minimum total score of 50 comprising at least a peptide match of ion score more than 20 were arbitrarily set as threshold for acceptance (Seo et al., 2008).

In vitro interaction of GAPDH – Apo Tf by solid phase assays: In vitro interaction of GAPDH and Apo Tf was analyzed by ELISA and surface plasmon resonance (SPR) based
affinity interaction analysis by standard procedures as described previously (Raje et al., 2007). Briefly, polystyrene ELISA plate wells were coated overnight at 4°C using 150 nM/well of rabbit muscle GAPDH in PBS and blocked with 5% BSA for 24 hrs at 4°C. Wells were then incubated with different concentrations of Apo Tf in PBST (PBS + 0.05% Tween-20) containing 0.5% BSA for 24 hrs at 4°C. After extensive washing, the bound Apo Tf was detected by incubation with rabbit anti transferrin antibody followed by goat anti rabbit-HRP. TMB H₂O₂ substrate for ELISA (Bangalore Genei) was used to develop the reaction and OD was measured at 450 nm. As a positive control for Apo Tf, a set of wells were coated with Apo Tf alone. Controls were also set up to determine the nonspecific interaction of (i) anti transferrin antibody with GAPDH, (ii) anti transferrin antibody with BSA, (iii) Tf with BSA, (iv) secondary antibody with GAPDH and (v) secondary antibody with BSA. Each set consisted of four wells in replicates. For SPR assay biotinylated Apo Tf was immobilized on sensor chip SA (Biacore) as per manufacturer’s instructions (Biacore 3000). Subsequently, increasing concentrations of GAPDH were used as an analyte in HBS-EP buffer (Biacore) at pH 7.4. Regeneration of sensor chip was carried out using 250 mM NaOH. K_{on}, K_{off} and K_D value were determined by using 1:1 binding model Biacore 3000® evaluation software (Raje et al., 2007).

Apo Tf binding to iron loaded cells is pronase sensitive and specific: Excess iron treated J774 cells were pretreated with 0.1% pronase(Roche) or control buffer for 20 min at 4°C, subsequently cells were washed with SFM and stained with Apo Tf Alexa647 at 4°C for 1 hr. Non specific binding was evaluated by incorporating 200x unlabeled Apo Tf in the staining mixture. All samples were analyzed by FACS.

Binding of Apo Tf to cells is saturable: The characteristics of Apo Tf binding onto cells was assessed essentially as described previously(Kumar et al., 2012). Briefly, J774 cells were cultured in 96 well plates (2x10⁴ cells/well) and loaded with iron by incubation with100µM FeCl₃ for 24 hours. Subsequently, cells were incubated with increasing concentrations of biotinylated Apo Tf, either alone or in presence of 200X unlabeled Apo Tf (to evaluate nonspecific interaction), for 2hr at 4°C. This was followed by incubation with 1:5000 diluted Streptavidin-HRP for 45 min at 4°C. The reaction was developed with TMB H₂O₂. To determine binding affinity, Apo Tf concentration versus OD data was fitted by nonlinear regression for, one site total saturable binding, using GraphPad® software.

Co-localization of cell surface proteins by confocal microscopy: Excess iron treated J774 cells were washed and blocked with neutral buffer containing 5% each of, foetal calf serum and normal human serum. For cell surface colocalization of GAPDH and Apo Tf, cells were
incubated with 1µg of monoclonal anti GAPDH (Calbiochem) followed by rabbit anti mouse Alexa 568 (Molecular Probes) and 10µg of Apo Tf FITC. For simultaneous co-localization of GAPDH, Apo Tf and ferroportin, 1:100 diluted goat anti ferroportin antibody (Santacruz) was included in the above antibody incubation mixture. After extensive washing, cells were incubated with rabbit anti mouse Alexa 568(for detection of GAPDH) followed by mouse anti goat FITC (Santacruz) and 10µg of Apo Tf Alexa647. All antibody incubations were carried out sequentially at 4°C. Finally, cells were washed, fixed in 1% paraformaldehyde and imaged in a confocal microscope (Nikon A1R) using a 63 X oil immersion objective and 1 Airy unit aperture as described previously (Sheokand et al., 2013). Co-localization of signals was visualized manually in the merge image and Pearson’s correlation coefficient calculated using Nikon NIS-Elements® software.

Interaction of GAPDH-Apo Tf & GAPDH-Ferroportin: Interaction of proteins on surface of iron loaded J774 cells was assessed by confocal microscopy based co-localization, co-immunoprecipitation and acceptor photobleaching FRET analysis as described previously (Raje et al., 2007; Sheokand et al., 2013). For Co-IP of GAPDH and Apo Tf, 2x10^7 iron treated J774 cells were incubated with 500 µg of biotinylated Apo Tf in 1 ml FACS buffer for 1 h on ice. Controls were set in parallel wherein incubation of cells with biotinylated Apo Tf was omitted. Cells were washed and processed for preparation of the membrane protein fractions. Co-immunoprecipitation was performed from these fractions using Streptavidin Magnabeads® (Polysciences) as per the manufacturer’s instructions. Beads were boiled in SDS sample buffer, and eluted proteins were analyzed by western blot using monoclonal anti-GAPDH antibody. For Co-IP of GAPDH and Ferroportin, iron treated J774 cells were processed for preparation of the membrane protein fraction as above. Co-immunoprecipitation was performed using monoclonal anti GAPDH immobilized onto anti-mouse IgG Magna beads® (Polysciences). Negative control was run in parallel wherein membrane fraction was co-immunoprecipitated using isotype control mouse IgG immobilized on anti mouse IgG magna beads. Beads were boiled in SDS sample buffer, and eluted proteins were analyzed by western blot using anti ferroportin antibody.

In vivo study in rodent model: Male Sprague–Dawley rats (150–170 g) of 4-6 weeks age were administered with a total of 700 mg iron dextran i.p over a 16-week period essentially as described earlier (Brown et al., 2007) and approved by the institutional animal ethics committee. Groups of animals were sacrificed over a period of 1 week. Hepatocytes, peritoneal macrophages and enterocytes were isolated and stained for surface GAPDH and
Apo Tf Alexa 647 and analyzed by FACS. Liver tissue and serum samples were assayed for iron using Quantichrome iron assay kit.

**Chromogenic iron release assay:** J774 cells were treated with 100µM FeCl₃ for 24 hr and harvested. Aliquots of 1X10⁵ cells were incubated with 100µl SFM or SFM supplemented with 0.3mg Apo Tf at 37°C for 1 hr. Subsequently, cells were centrifuged (500g, 5 min) to collect supernatant. The cell pellet was washed 3X with neutral buffer (20mM HEPES, 150mM NaCl, 5mM KCl and 1mM each of CaCl₂ and MgCl₂). Both, cell pellet and respective cell supernatant were digested with 5%HNO₃ at 80°C for 2 hr to release iron. Samples were concentrated in a speed vac concentrator and iron estimation was performed using a Quantichrome® iron assay kit (Bioassay systems) as per manufacturer’s instructions.

**Iron release assay using radioactive iron:** Cells were loaded with iron either by incubation with FeCl₃ or erythrophagocytosis (spleen macrophage, THP1 and J774) in complete medium spiked with 500nM of ⁵⁵FeCl₃ (ARC) at 37°C. Controls were set up in parallel with complete medium containing 500nM of ⁵⁵FeCl₃. After 12 hrs cells were washed extensively with SFM and incubated with 200µl of SFM containing 2 mg/ml Apo Tf or with only SFM (as control). Subsequently the cell free supernatants were collected and assayed for presence of ⁵⁵Fe by liquid scintillation counting.

**Sequestration of exported iron by Apo Tf:** J774 cells cultured in 24 well plates (3x10⁵/well) were iron loaded by FeCl₃ treatment along with 500nM ⁵⁵FeCl₃ as for the iron release assays described above. Cells were then incubated with 0.5 mg/ml biotinylated Apo Tf at 37°C for 1 hr in SFM. Subsequently the supernatant was collected and biotinylated Apo Tf was captured using streptavidin Magna beads. Precipitated samples were blotted on to nitrocellulose membrane and radioactive iron was detected using a phosphoimager (Fujifilm FLA-9000).

**Statistical analysis:** All statistical analysis was carried out using students unpaired t test.

**Acknowledgements**

Mr. Anil Theophilus & Dr. Subash Pawar are acknowledged for technical assistance. N.S., H.M. and A.S.C. received fellowships from CSIR, UGC and DBT respectively. Financial support of CSIR and DST is acknowledged. The authors have no conflicting financial interests. This is IMTECH communication No. 0140/2013.

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Figure legends

Figure 1: Modulation and characterization of surface GAPDH upon iron loading of cells. (A) J774 cells were cultured in 100µM FeCl3 supplemented medium for increasing time intervals and checked for change in surface as well as intracellular expression of GAPDH by FACS analysis. Extracellular GAPDH was also monitored for ectoenzyme activity. Surface and intracellular GAPDH, data is presented as % mean fluorescence intensity of control samples, p <0.005, *(p >0.05), n=10^4. For ectoenzyme activity, data is presented as % OD at 340 nm of control sample, (p<0.0005), n=4. Experiments were repeated multiple times. (B) J774 cells were treated with increasing concentrations of iron upto 100 µM FeCl3 and surface expression of GAPDH was evaluated. Data is presented as % mean fluorescence intensity of control samples, p <0.0001, n=10^4. (C) The extent of increase in cell surface GAPDH is significantly more upon iron loading of cells as compared to iron depletion. J774 cells were treated with either 100µM FeCl3 or 100µM DFO for 24 hours before FACS based analysis of surface GAPDH p <0.0001, n=10^4. (D&E) GAPDH is present in both DRM and non DRM membrane fractions in control as well as iron loaded J774 cells (p<0.0001), statistical analysis was carried out using Mean fluorescence intensity (MFI), n=10^4. (F) Western blot of 2D-gel electrophoresis separated GAPDH isoforms from membrane fractions of iron loaded (100µM FeCl3) and iron depleted (100µM DFO treated) J774 cells. Please also see Fig S1.

Figure 2: LC-MS/MS analysis of membrane GAPDH from iron depleted and iron loaded cells. J774 cells were cultured in 100µM FeCl3 or 100µM DFO supplemented medium for 24 hrs and membrane fractions purified. Membrane proteins from both samples were extracted and run on 12% SDS-PAGE. Bands corresponding to GAPDH were excised
and trypsin digested. Peptide analysis by Mascot following LC-MS/MS confirmed proteins to be GAPDH. The MOWSE score was >700 with sequence coverage >80% in both cases. (A) PTMs observed in GAPDH recruited upon iron depletion in J774 cells. (B) PTMs observed in GAPDH recruited upon iron overload in J774 cells. (C) Comparative analysis of various PTMs and corresponding modified residues in GAPDH recruited under both conditions.

**Figure 3: Binding of Apo Tf iron loaded cells is selectively enhanced.** (A) Significant decrease in cell surface Holo Tf Alexa 647 binding by iron loaded J774 cells (p<0.05), n=10^4. (B) Apo Tf Alexa 647 binding is increased on the surface of iron loaded J774 cells (p<0.0001), n=10^4. (C) The increase in Apo Tf binding progresses with time of exposure to iron. J774 cells were treated with 100µM FeCl₃ for increasing time and binding of Apo Tf Alexa 647 was measured. Data is presented as % of mean fluorescence intensity of control samples (p <0.005), n=10^4. (D) Concentration dependent binding of Apo Tf on the surface of iron loaded J774 cells. Results are expressed as concentration of biotinylated Apo Tf versus OD (450nm) ± SD. (E&F) Binding of Apo Tf to surface of iron treated J774 cells is specific (E) and pronase sensitive (F) p<0.0001,n=10^4. All experiments were repeated three times.

**Figure 4: Cell surface GAPDH interacts with Apo Tf on cell surface** (A) Colocalization of GAPDH and Apo Tf-FITC on the surface of excess iron treated J774 cells using confocal microscopy, Pearson’s correlation coefficient is 0.94, also see Fig S2C&D. GAPDH was detected with mouse monoclonal anti GAPDH and anti mouse Alexa 568. (B) GAPDH on the surface of excess iron treated J774 cells interacts with Apo Tf as seen by FRET signal which is represented by an increase (arrowheads) in donor signal (anti-GAPDH TRITC) upon bleaching of acceptor (Apo Tf Alexa 647), Bar = 5µ. (C) FRET efficiency [signal intensity of (Donor_post Bleach - Donor_pre Bleach)/ Donor_post Bleach x 100] was compared to control where instead of Apo Tf Alexa 647 unrelated goat IgG Alexa 647 was used, p<0.0001, n=25. (D) Excess iron treated J774 cells were incubated with biotinylated Apo Tf at 4°C, membrane fraction prepared and subjected to co-immunoprecipitation using streptavidin magna beads. Interaction between GAPDH and Apo-Tf was confirmed by western blot using monoclonal anti GAPDH antibody. Control was run in parallel wherein incubation of cells with biotinylated Apo Tf was omitted. Please also see Fig S2.

**Figure 5: Modulation of surface GAPDH and Apo Tf binding in cells isolated from chronic iron loaded SD rats.** (A) Primary hepatocytes and peritoneal macrophages isolated from iron loaded rats demonstrate increase in both, surface GAPDH expression as well as Apo Tf binding as compared to cells isolated from control rats, p<0.05 (hepatocytes), p<0.002 (peritoneal macrophages) n=3. Enterocytes isolated from iron loaded rats showed a
decrease in both surface GAPDH expression and Apo Tf binding in comparison to cells isolated from control rats, p<0.001, n=3. From each test animal 10^4 cells were analyzed by flow cytometry for each parameter separately and results are presented as mean fluorescence intensity (MFI± SD). Data is representative of 5 independent experiments (See Fig S3 for results of all 5 groups). (B) Correlation between change in expression of surface GAPDH and Apo Tf binding on cells from all iron loaded animals. Please also see Fig S3.

**Figure 6: GAPDH facilitates iron export via Apo Tf in iron loaded cells.** (A&B) Cell lines as well as rat primary cells were iron loaded using either 100µM FeCl₃ treatment (A) or EPG (B) in a media spiked with ^{55}Fe for 12 hours. Subsequent incubation of cells with Apo Tf resulted in enhanced iron export into the incubation medium as compared to cells where Apo Tf was omitted, p<0.0005, *p<0.001, # p>0.05, n=4. Data is presented as percentage of CPM in incubation medium of cells incubated without Apo Tf ± SD, each experiment was repeated multiple times. (C) The effect of Apo Tf in enhancing iron exit is dose dependent. J774 cells treated with 100µM FeCl₃ (spiked with ^{55}FeCl₃) were incubated with increasing concentrations of Apo Tf or 4mg/ml Holo Tf (the maximum concentration of Apo Tf tested) and iron exit into incubation media was quantified, p<0.0001, # p>0.05, n=4. Data is presented as percentage CPM in incubation medium of cells incubated without Apo Tf ±SD.

(D) Iron released by cells is loaded onto Apo Tf. J774 cells loaded with iron as above were incubated with 0.5 mg/ml of biotinylated Apo Tf, after immunoprecipitation of Apo Tf from cell supernatant with streptavidin magna beads the captured radioactive iron was visualized on nitrocellulose membrane by phosphoimager. Negative control was run in parallel where incubation with biotinylated Apo Tf was omitted. Please also see Fig S4.

**Figure 7: GAPDH and ferroportin interact at surface of iron loaded J774 cells.** (A) Colocalization of GAPDH, ferroportin and Apo Tf on the surface of iron treated J774 cells by confocal microscopy. Pearson’s correlation coefficient for ferroportin with GAPDH and Apo Tf is 0.92 and 0.95 for GAPDH with Apo Tf. (B) GAPDH on the surface of iron loaded J774 cells interacts with ferroportin as observed by FRET signal (arrows) represented by an increase in donor signal (ferroportin) upon bleaching of acceptor (GAPDH), bar = 5µ. (C) GAPDH-Ferroportin FRET control where instead of specific antibody against ferroportin control goat IgG was utilized for donor staining. Acceptor is same as in Fig 6B, bar = 10µ. (D) FRET efficiency (calculated as described in legend 3C) as compared to non specific control (instead of goat anti ferroportin antibody normal goat IgG was utilized), p<0.0001, n=15. (E) Co-immunoprecipitation of ferroportin and GAPDH from cell surface of iron loaded J774 cells. Membrane fraction was prepared from iron treated J774 cells (2x10^7 cells)
and immunoprecipitated using mouse monoclonal anti GAPDH immobilized onto anti-mouse IgG magna beads. Interaction with ferroportin was confirmed by western blot developed with goat anti ferroportin antibody. Negative control was run in parallel wherein membrane fraction was co-immunoprecipitated using isotype control mouse IgG immobilized on anti mouse IgG magna beads while membrane fraction of iron loaded J774 cells served as positive control for ferroportin. (F) Higher order multifunctionality of GAPDH a model for its role in cellular iron efflux.

Table I

Fold change in surface expression of GAPDH and Apo Tf binding upon iron overload as compared to control cells at 24 hrs.

<table>
<thead>
<tr>
<th>Experimental iron Overload</th>
<th>Cell type</th>
<th>Surface GAPDH</th>
<th>Apo Tf binding</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ex Vivo FeCl₃</td>
<td>Rat hepatocytes</td>
<td>2.7</td>
<td>1.84</td>
</tr>
<tr>
<td></td>
<td>Rat peritoneal macrophages</td>
<td>4</td>
<td>2.17</td>
</tr>
<tr>
<td></td>
<td>Rat spleen macrophages</td>
<td>1.83</td>
<td>1.45</td>
</tr>
<tr>
<td></td>
<td>Rat enterocytes</td>
<td>0.75</td>
<td>0.74</td>
</tr>
<tr>
<td></td>
<td>J774</td>
<td>3.38</td>
<td>2.77</td>
</tr>
<tr>
<td></td>
<td>RAW</td>
<td>1.78</td>
<td>1.59</td>
</tr>
<tr>
<td></td>
<td>THP1</td>
<td>2.41</td>
<td>1.94</td>
</tr>
<tr>
<td></td>
<td>GAPDH knockdown THP1</td>
<td>ND*</td>
<td>ND*</td>
</tr>
<tr>
<td>Erythrocyte phagocytosis</td>
<td>Rat spleen macrophages</td>
<td>1.46</td>
<td>1.56</td>
</tr>
<tr>
<td></td>
<td>J774</td>
<td>2.4</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>THP1</td>
<td>1.77</td>
<td>1.52</td>
</tr>
<tr>
<td></td>
<td>GAPDH knockdown THP1</td>
<td>ND*</td>
<td>ND*</td>
</tr>
<tr>
<td>In vivo Fe dextran</td>
<td>Rat peritoneal macrophages</td>
<td>1.48</td>
<td>1.62</td>
</tr>
<tr>
<td>Erythrocyte phagocytosis</td>
<td>Rat peritoneal macrophages</td>
<td>2.06</td>
<td>5.5</td>
</tr>
</tbody>
</table>

Table I: Different cell types were subjected to an iron overload either ex vivo (by FeCl₃ or RBC phagocytosis for 24 hrs) or by i.p. injection of Fe dextran/opsonized erythrocytes into male Sprague Dawley rats. Peritoneal macrophages were isolated at 24 hrs. Controls were set in parallel (see supplementary methods for details). Surface expression of GAPDH and Apo Tf binding was evaluated by Flow cytometry, p < 0.0001 in all cases except #, ND Not Detectable, n=10⁴ in each case, all experiments repeated at least 3 times.
Membrane GAPDH from iron depleted cells

Membrane GAPDH from iron treated cells

<table>
<thead>
<tr>
<th>PTMs</th>
<th>GAPDH (iron depleted)</th>
<th>GAPDH (iron treated)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methylation(D,E)</td>
<td>2 [D37, E333]</td>
<td>3 [D283, D294, E333]</td>
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<tr>
<td>Oxidation(M,C)</td>
<td>7[M41, M103, M128, M131, C154, M173, M329]</td>
<td>3[M128, M173, M329]</td>
</tr>
<tr>
<td>Phosphorylation(S,T,Y)</td>
<td>1[T244]</td>
<td>ND*</td>
</tr>
<tr>
<td>Acetylation(K)</td>
<td>2[K137, K143]</td>
<td>ND*</td>
</tr>
<tr>
<td>Nitrosylation(C)</td>
<td>2[C154, C245]</td>
<td>1[C154]</td>
</tr>
<tr>
<td>Propionamide(C)</td>
<td>2[C245, C282]</td>
<td>2[C245, C282]</td>
</tr>
<tr>
<td>Pro-pyro glu(P)</td>
<td>3[P127, P234, P236]</td>
<td>1[P34]</td>
</tr>
<tr>
<td>Succinylation(K),</td>
<td>ND*</td>
<td>ND*</td>
</tr>
<tr>
<td>ADP-ribosylation(C),</td>
<td>ND*</td>
<td>ND*</td>
</tr>
<tr>
<td>Palmitoylation(C)</td>
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<td>GPI anchor(protein C term),</td>
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<tr>
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<td>Myristoylation(Nterm G),</td>
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<tr>
<td>Pyro-Glu(E, Q)</td>
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<td>ND*</td>
</tr>
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</table>

* Not Detected
Fig 5

A

Surface GAPDH expression

Hepatocytes

MFI ± SD

Control
High iron

Apo Tf binding

MFI ± SD

Control
High iron

B

% Increase in Apo Tf Binding

r = 0.67, p<0.01

Hepatocytes

Peritoneal Macrophages

Enterocytes

% Increase in cell surface GAPDH
Figure 7

Higher order Moonlighting behaviour by GAPDH a novel player in maintaining cellular iron homeostasis.