ADHFe1: a novel enzyme involved in retinoic acid-dependent Hox activation

YEHUDA SHABTAI*, NATALIE SHUKRUN* and ABRAHAM FAINSOD*

Department of Developmental Biology and Cancer Research, Institute for Medical Research Israel-Canada, Faculty of Medicine, The Hebrew University of Jerusalem, Israel

ABSTRACT Retinoic acid (RA) signaling is a central pathway regulating anterior-posterior patterning of the embryo through its targets, the Hox genes. RA is produced by two sequential oxidations from vitamin A (retinol) and this biosynthesis has to be regulated temporally, spatially and quantitatively. Mining Xenopus embryonic expression databases identified a novel component of the RA metabolic network, ADHFe1. Using Xenopus laevis embryos as our experimental system we determined the temporal and spatial pattern of AdhFe1 expression. Gain- and loss-of-function of ADHFe1 were induced to study its function and the regulation of the AdhFe1 gene by RA was studied. Expression analysis localized the ADHFe1 protein to the late Spemann’s organizer, the trunk organizer. Subsequently, ADHFe1 can be detected in the prechordal mesoderm, the notochord and the lateral plate mesoderm. Manipulation of ADHFe1 levels affects the normal Hox gene expression. The effects of ADHFe1 manipulation can by rescued by increasing the levels of RA or its biosynthesis. Expression of the AdhFe1 gene is regulated by RA itself. ADHFe1 is an enzyme active already during gastrula stages and the protein is still present during neurula stages. ADHFe1 regulates the expression of the Hox genes during the early patterning of the trunk. The effect of ADHFe1 on Hox expression is mediated through regulation of RA levels. ADHFe1 probably reduces retinaldehyde to retinol thereby restricting the availability of retinaldehyde, the substrate needed by retinaldehyde dehydrogenases to produce RA making it a novel regulator of RA concentrations in the embryo and RA homeostasis.

KEY WORDS: anterior-posterior patterning, Xenopus, alcohol dehydrogenase, hydroxyacid-oxoacid transhydrogenase

Introduction

Retinoic acid (RA) signaling is one of the central regulatory pathways active during early embryogenesis. It plays a pivotal role in patterning the embryo along the anterior-posterior axis by dose-dependent activation of Hox genes as well as by regulating somitogenesis. As such, this signaling pathway becomes activated in multiple sites during embryogenesis and in multiple instances in parallel. This parallel activity in different tissues and cell types, under differing conditions requires precise spatial, temporal and quantitative regulation. Several basic regulatory mechanisms cooperate to achieve this goal. RA signaling itself regulates the expression of multiple enzymes involved in RA biosynthesis and catabolism (Kedishvili, 2013). Enzymes like CYP26 of the cytochrome p450 family metabolize RA to render it biologically inactive and promote its degradation (Kedishvili, 2013). Also, expression of the nuclear receptors mediating the gene regulatory effects of RA, i.e. retinoic acid receptors (RAR) and retinoid X receptors (RXR), is tightly regulated in time and space (Dollé, 2009).

RA is produced from vitamin A (retinol, ROL) through two sequential oxidation steps (Duester, 1996; Kedishvili, 2013). The first oxidation reaction is catalyzed by enzymes with alcohol dehydrogenase (ADH) activity and produces retinaldehyde (RAL). Subsequently,
the RAL is oxidized by enzymes with aldehyde dehydrogenase (ALDH) activity to produce RA. The main members of the ALDH family producing RA are also known as retinaldehyde dehydrogenases (RALDH). The oxidation of ROL to RAL is performed by two families of enzymes, either members of the medium-chain ADH family or enzymes belonging to the short-chain dehydrogenase/reductase (SDR) family (Duester, 1996; Kedishvili, 2013).

During embryogenesis the activation of RA signaling correlates with the zygotic expression of the *Raldh2* gene (Ang, Duester, 1999; Chen *et al.*, 2001). In *Xenopus* embryos *Raldh2* mRNA initially localizes to Spemann’s organizer on the dorsal lip of the blastopore (Chen *et al.*, 2001). The early requirement for RALDH2 activity is supported by the observation that mouse mutants in the *Raldh2* gene die soon after gastrulation (Niederreither *et al.*, 1999). In line with this reasoning, it has been shown that during late blastula stages the embryo is primed to activate RA signaling but still requires a RALDH activity (Ang, Duester, 1999). Which enzymes are involved in the ROL to RAL oxidation reaction is less clear. Studies in a number of vertebrate model systems have identified the SDRs, RDH10 and RDHE2 as endogenous retinol dehydrogenases that provide an early activity required to produce RAL (Belyaeva *et al.*, 2012; Rhinn *et al.*, 2011). In *Xenopus* embryos, *Rdh10* is expressed in a dorsal domain that partially overlaps with the domain of *Raldh2* expression (Strate *et al.*, 2009). *Rdhe2* is only zygotically transcribed from neurula stages onwards and does not qualify for the early function during gastrulation (Belyaeva *et al.*, 2012). Another member of the SDR family, DHRS3 (retSDR), has been described that overlaps both spatially and temporally in its expression with RALDH2 and RDH10 (Kam *et al.*, 2010). DHRS3 has been shown to play an important regulatory role by attenuating or restricting the RA signal (Billings *et al.*, 2013; Feng *et al.*, 2010). This enzyme mainly reduces RAL to ROL thus regulating the substrate availability for RALDH enzymes.

In order to identify additional enzymes possibly involved in gastrula stage RA metabolism, we searched the available *Xenopus* gene expression databases (Xenbase and others). We identified ADHFe1, as an enzyme expressed during mid gastrula stages beginning at stage 11 and continuing through neurula stages. *Xenopus* ADHFe1 protein and transcripts initially localize to the dorsal lip of the blastopore. Following invagination, they extend along the dorsal midline. During neurula stages the protein also localizes to the lateral plate mesoderm. Knock-down and overexpression experiments show that ADHFe1 is involved in the fine tuning of RA levels, using Hox gene expression as a readout. Loss-of-function resulted in upregulation of Hox transcription while ectopic expression compromised Hox activation. These results demonstrate that ADHFe1 is involved in RA-dependent anterior-posterior (AP) pattern formation, probably by performing the reduction of RAL to ROL, i.e.

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**Fig. 1.** *Xenopus laevis* ADHFe1 is extensively conserved among vertebrates. The full length open reading frame encoded by the *AdhFe1* gene was PCR cloned from embryonic *Xenopus laevis* RNA samples. *(A)* Protein sequence alignment of the *X. laevis* ADHFe1 protein to its orthologs from other vertebrates. Identical protein sequences are in gray boxes. The red line marks the human ADHFe1 sequence used to raise the polyclonal antibody. *(B)* Phylogenetic tree of the ADHFe1 protein family among representative vertebrates. The percent sequence identity to the *X. laevis* ADHFe1 sequence are shown.
by restricting the substrate availability for RALDH. Together, this work identified a novel regulatory component in the metabolism of RA during early embryogenesis.

Results

Identification and cloning of ADHFe1

In an effort to better understand the biosynthesis of RA during gastrulation, we focused our efforts on identifying members of the ADH and SDR enzyme families expressed from late blastula to early neurula stages. We performed extensive data mining of expression databases (Bowes et al., 2010; Tan et al., 2013; Yanai et al., 2011) to identify those members of the ADH and SDR families known to be expressed during these stages. Among the genes identified, we found AdhFe1 which encodes an enzyme that has never been linked to RA metabolism. To characterize this enzyme in the context of early embryogenesis, we PCR cloned the full length open reading frame of AdhFe1 from embryonic Xenopus laevis RNA samples (Fig. 1A). The predicted Xenopus laevis ADHFe1 protein is 463 amino acids in length. The Xenopus protein displays extensive homology to other vertebrate ADHFe1 family members (85% to 96% protein sequence identity; Fig. 1A,B), demonstrating a common evolutionary origin of this subfamily of the alcohol dehydrogenases (Fig. 1B).

Onset of embryonic ADHFe1 expression coincides with the main wave of Hox activation

To begin the characterization of the AdhFe1 gene we determined its temporal pattern of expression during early embryogenesis by quantitative real-time PCR (qPCR). RNA samples were collected from multiple embryonic stages from 16-cell stage (stage 5) to the initial neural tube stage (stage 19) (Nieuwkoop, Faber, 1967). Our results show that robust AdhFe1 transcription begins towards mid gastrula (stage 11; Fig. 2A). Expression of AdhFe1 peaks towards the end of gastrulation (stage 12.5) / beginning of neurulation (stage 13) and remains high throughout the rest of the stages tested. Before stage 11, AdhFe1 transcripts are basically undetectable. This temporal pattern of expression is in agreement with the parameters we employed to search the expression databases to identify putative novel components of the RA metabolic network. The expression of Rdh10 and Dhrs3 was also studied in the same samples. This comparative expression analysis revealed that both members of the SDR family were up-regulated at the onset of gastrulation before the initial transcription of AdhFe1 (Fig. 2A). Also, while Rdh10 and Dhrs3 are down-regulated towards the end of gastrulation, AdhFe1 remains strongly expressed during neurula stages (Fig. 2A).

To determine the spatial pattern of ADHFe1 activity we focused on the localization of the protein. To that end, a polyclonal antibody raised against a highly conserved human ADHFe1 peptide was used (Fig. 1A). To test the cross-reactivity and specificity of the anti-ADHFe1 antibodies, we transfected HEK293 cells with a pCS2-XADHFe1 plasmid to induce the expression of Xenopus AdhFe1. Co-transfection with a GFP expression plasmid was used to monitor transfection efficiency. RNA and protein extracts were prepared from experimental and control groups. Immunodetection of the expressed Xenopus ADHFe1 revealed that the protein was only detectable in cells transfected with the pCS2-XADHFe1 plasmid and not in the control transfected sample (Fig. 3A). The protein detected had an estimated molecular weight of about 50 KD which corresponds to the predicted size from the cDNA sequence. To further demonstrate that the protein detected is the product of the pCS2-XADHFe1 expression plasmid we analyzed the RNA produced in the transfected cells. For this reason, we analyzed by qPCR the total RNA of the transfected cells with primers specific for the Xenopus AdhFe1 transcript. The results show that only the cells transfected with the expression plasmid gave a robust signal while the control sample had no PCR product (Fig. 3B). These results together show that the antibody against the human ADHFe1 can robustly detect the Xenopus protein product. Embryos from defined stages were stained with the anti-ADHFe1 antibody to determine the spatial pattern of ADHFe1 expression. During the initial stages of ADHFe1 expression (stage 11), the protein...
protein encoded by this gene localized to the dorsal blastopore lip, i.e. the late Spemann’s organizer (Fig. 4A). Towards the end of gastrulation (stage 12), ADHFe1 protein was found at the dorsal midline (Fig. 4B), suggesting that ADHFe1 positive cells invaginated during late gastrulation. To corroborate the dorsal midline expression of ADHFe1, we focused on localizing the mRNA of this gene. Stage 12 embryos were dissected into dorsal, lateral and ventral regions and RNA was prepared from each region. qPCR analysis with Xenopus AdhFe1 specific primers revealed that most transcripts of this gene localize to the dorsal region (Fig. 2B). Analysis of the localization of chordin, MyoD and sizzled transcripts revealed the expected dorsal, lateral/ventral and ventral distribution of transcripts respectively (Fig. 2 C-E). These results further support the dorsal localization of the ADHFe1 expression during gastrulation.

During mid neurula stages (stage 17), staining along the dorsal midline was still evident (Fig. 4D). Bisecting of the embryos confirmed the localization of the ADHFe1 protein along the axial mesoderm, in the notochord and the prechordal mesoderm (PCM) (Fig. 4E). At stage 17 as well as stage 22, an additional ADHFe1 expression domain was detected (Fig. 4 C,D,F,G). This novel expression localized to the lateral plate mesoderm (LPM) as evidenced from the bisected embryos (Fig. 4H). Interestingly, this localization of the ADHFe1 protein abuts the CNS including the rostral region (Fig. 4C,D). Analysis of earlier developmental stages revealed that the LPM expression began with the transition from gastrula to neurula stages (stage 12; Fig. 4B).

**Developmental and biochemical functions of ADHFe1**

To investigate the biochemical function of ADHFe1 in the metabolism of RA we performed knock-down and gain-of-function experiments. To monitor the effect on RA biosynthesis, the changes in expression of 3’ Hox genes, which are highly sensitive to RA levels, was determined (Cunningham, Duester, 2015). An antisense morpholino oligonucleotide (MO) was designed to knock-down ADHFe1 levels (Heasman, 2002). To test the MO efficiency we constructed an expression plasmid with the AdhFe1 MO target sequence upstream of the GFP sequence. Embryos were co-injected with the ADHFe1 target/GFP expression vector and the AdhFe1-specific MO. At stage 11 the GFP-dependent fluorescence was analyzed (Suppl. Fig. S1). While all the embryos injected with the AdhFe1 MO lost fluorescence (n=20; Suppl. Fig. S1F), all embryos co-injected with the control MO retained their fluorescence (n=20; Suppl. Fig. S1E). The fluorescence of embryos injected with a control GFP expression plasmid containing the Raldh2 MO target sequence was not affected by co-injection of the AdhFe1 MO (n=20; Suppl. Fig. S1C) or the control MO (Suppl. Fig S1B). These results strongly support the specificity of the AdhFe1 MO. To prove the MO specificity beyond doubt, ADHFe1 protein levels were analyzed in morphants by immunodetection. AdhFe1 MO was injected into one blastomere of the 2-cell embryo together with a lineage tracer. During neurula (stage 17), embryos were processed for immunodetection of the endogenous ADHFe1 protein (purple) and the lineage tracer (turquoise) (Fig. 3C,D). The ADHFe1 MO drastically reduced the amount of ADHFe1 protein.
ADHFe1 regulates RA homeostasis in the injected side (Fig. 3D) while injection of the control MO had no effect on the protein levels (Fig. 3C). These results demonstrate the specificity of the AdhFe1 MO and the anti-ADHFe1 antibody.

To characterize the function of ADHFe1 in the context of RA biosynthesis, embryos were injected with increasing amounts of MO (0.8-3.2 pmol) and the effect was studied by monitoring the change in Hox expression. All four Hox genes monitored, HoxA1, HoxB1, HoxB2 and HoxB4 exhibited a dose-dependent response to the AdhFe1 MO (Fig. 5A). Interestingly, all four Hox genes were up-regulated as a result of the ADHFe1 knock-down, suggesting that this enzyme functions primarily to reduce the level of RA in the embryo.

We turned to overexpression (OE) of AdhFe1 to corroborate the effect of this enzyme on Hox expression. Embryos were injected with the AdhFe1 expression plasmid (pCS2-XADHFe1; 160 ng/embryo) and RNA was prepared for qPCR analysis of Hox expression at stage 11. In agreement with the knock-down results, ADHFe1 OE resulted in strong down-regulation of HoxA1, HoxB1, HoxB2 and HoxB4 expression (Fig. 5B). This result further supports the notion that ADHFe1 activity promotes a decrease in RA levels.

To further explore this effect of ADHFe1 on the regulation of Hox gene expression and to determine whether it is mediated through RA signaling we designed rescue experiments. We reasoned that if ADHFe1 regulated RA levels by reducing RAL to ROL, i.e. by reducing the substrate availability for RALDH enzymes, then supplementation with RA precursors should overcome this activity. To test this hypothesis, embryos were injected with the AdhFe1 expression plasmid and supplemented with either ROL (10 μM) or RAL (1 μM). Analysis of HoxA1 and HoxB1 expression showed that ADHFe1 OE alone resulted in strong down-regulation of their expression (Fig. 5C,D). Treatment of the control embryos with ROL or RAL, in contrast, resulted in the expected up-regulation in HoxA1 and HoxB1 expression as a result of the promotion in RA biosynthesis (Fig. 5C,D). In agreement with our conclusion, supplementation with the RA precursors, RAL and ROL, overcame the down-regulatory effect of ADHFe1 OE, providing a partial rescue of expression levels (Fig. 5C,D). Together, these results strongly suggest that the effect of ADHFe1 on Hox expression is mediated through regulation of RA biosynthesis.

As mentioned above, RA feedback transcriptional regulation is a hallmark of genes encoding enzymes involved in RA metabolism. To test whether this holds for AdhFe1 as well, we determined if AdhFe1 gene expression is also under RA regulation. The levels of RA in the embryo were manipulated in two ways: RA biosynthesis was compromised by inhibiting RALDH with DEAB or citral. Increased RA levels were achieved by precursor supplementation (ROL or RAL) or actual RA treatment. During mid and late gastrulation, the levels of AdhFe1 expression were determined by qPCR. To...
asses the effect of the treatments on RA levels we determined the change in HoxB1 expression. Inhibition of RA biosynthesis with DEAB or citral severely reduced the expression of HoxB1 while ROL, RAL or RA supplementation increased the expression of this gene as expected (Fig. 6A). Analysis of AdhFe1 expression at stage 11 showed the inverse response to HoxB1 (Fig. 6B). This set of experiments suggest that RA signaling performs a negative regulatory role on AdhFe1 expression. Interestingly, when treated embryos were allowed to develop to late gastrula (stage 12) we identified a change in the regulation of AdhFe1. Any treatment that changes the level of RA, negatively affected the expression of AdhFe1 (Fig. 6C) showing a simultaneous requirement for RA and negative regulation by high levels. Taken together, we have identified a novel enzyme involved in RA metabolism during early embryogenesis, which is required for the correct temporal and spatial induction of Hox gene expression, i.e. setup of the Hox code and the resulting anterior-posterior patterning of the embryo.

Discussion

In this study, we have demonstrated a novel function of AdhFe1 in the regulation of RA homeostasis during early embryonic development. AdhFe1 has thus far not been analyzed in the context of vertebrate embryogenesis. A number of studies are, however, available on the function of this enzyme in other contexts. AdhFe1 (EC 1.1.99.24) is a member of the alcohol dehydrogenase family of enzymes that are iron-activated. This enzyme is also known as an hydroxyacid-oxoacid transhydrogenase involved in the oxidation of 4-hydroxybutyrate in vertebrates (Kardon et al., 2006). Although only partially characterized, expression of the AdhFe1 gene has been linked to differentiation of adipose tissue (Kim et al., 2007). Multiple studies have identified the AdhFe1 promoter as undergoing hypermethylation in colorectal carcinomas (Naumov et al., 2013) and its expression level was positively correlated to the differentiation state of colorectal tumors (Tae et al., 2013). These additional studies propose a link between AdhFe1 activity levels and the differentiation state of cells and tissues. Our work identifies AdhFe1 activity very early during embryogenesis when the germ layers begin to differentiate and patterning along the early embryonic axes takes place.

The initial localization of the AdhFe1 transcripts and protein product to the late Spemann’s organizer is of particular interest as this region acts as the so-called “trunk-tail organizer” (Niehrs, 2004). The trunk organizer is responsible for the formation and specification of embryonic regions caudal to the head domain. The vertebrate trunk domain is one of the main regions patterned by differential Hox gene activity (Durston, Zhu, 2015; Gaunt, 2015). Our results show that manipulation of AdhFe1 activity levels leads to abnormal expression of the Hox genes. Therefore, expression of AdhFe1 in the trunk organizer suggests a regulatory interaction with the Hox genes, a conclusion supported by our experiments. Future work will show to what extend the realization of the Hox code is dependent on AdhFe1, whether this enzyme merely fine-tunes RA levels or whether it may play a more active role in setting up the correct RA concentration along the AP axis.

Although AdhFe1 has been linked to a number of disease and differentiation processes, its biochemical function has only been partially characterized. The regulatory interaction between ADHFe1 activity and Hox expression levels provides an important insight for a novel activity of this enzyme. The Hox genes are some of the best characterized downstream targets of RA signaling (Parker et al., 2016). The changes in Hox gene expression following manipulation of AdhFe1 activity levels are consistent with changes in RA levels in the embryo. Although AdhFe1 has been described as an hydroxyacid-oxoacid transhydrogenase (Kardon et al., 2006), a role as an NAD(P)+-dependent alcohol dehydrogenase has also been proposed (Deng et al., 2002). As such, based on substrate specificity, this enzyme could perform the oxidation of ROL to RAL. Surprisingly, the response of the Hox genes to AdhFe1 manipulation was consistent with the opposite, a reduction in RA levels. This observation suggests that AdhFe1 also catalyzes the reduction of RAL to ROL, at least in the context of the early embryo. This conclusion was further supported experimentally by demonstrating that increasing ROL or RAL levels in the embryo rescued the effects of AdhFe1 overexpression. These results link AdhFe1 to RA biosynthesis and suggests that this enzyme plays a role in the homeostatic regulating of RA levels.

Our results place ADHFe1 as a regulator of RA levels specifically during gastrula stages. The reduction of RAL to ROL would result in a reduced substrate availability for the RALDH, the enzyme which oxidizes RAL to RA (Shabtai et al., 2016). We have recently demonstrated that RA is not impacting on RALDH2 activity, i.e. that this enzyme, which performs the last step in RA biosynthesis, is devoid of a negative feedback regulatory loop (Shabtai et al., 2016).
Two members of the RALDH family are known to be expressed in Spemann’s organizer during gastrula stages, Raldh2 and Raldh3 (Chen et al., 2001; Lupo et al., 2005). Two additional members of the RA metabolic network have been described in this region, Rdh10 and Dhrs3 (Kam et al., 2010). DHR3 is of particular interest as it has shown to perform a homeostatic regulatory function on RA levels (Feng et al., 2010) similar to the one we propose here for ADHFe1. Together, these data suggest that RA biosynthesis is under tight enzymatic regulation to achieve accurate signaling levels. Dissection of individual enzyme contributions necessary to achieve robust RA-dependent embryonic patterning awaits further characterization in vertebrate embryos.

Materials and Methods

Embryo culture and treatments

Xenopus laevis frogs were purchased from NASCO (Fort Atkinson, WI). Experiments were performed after approval and under supervision of the Institutional Ethics Committee. Embryos were obtained by in vitro fertilization, incubated in 0.1% MBSH and staged according to Nieuwkoop and Faber (1967). For microinjection, 2- to 4-cell embryos were injected radially or on one side. For lineage tracing, the injected material was co-injected with FITC-dextran (M, 70,000) 10 pg/embryo (Sigma), subsequently detected either microscopically or with anti-fluorescein antibodies (1:5000, rabbit polyclonal antibody (1:500; PA5-31416; Thermo Fisher Scientific) was used. Results were calculated using the CP method. All qPCR primers used are listed in Table 1.

Western blot analysis

A rabbit polyclonal antibody raised against amino acids 110-335 of the human ADHFe1 (PA5-31416; Thermo Fisher Scientific) was used to detect Xenopus laevis protein. Protein lysates for Western blots were prepared by homogenizing embryos or HEK293 cells in ice-cold lysis buffer (10 mM Tris-Cl pH 8.0, 1 mM EDTA, 0.5 mM EGTA, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS, 140 mM NaCl) supplemented with protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 mM pepstatin, and 10 μg/ml aprotinin). Homogenates were cleared by centrifugation at 14,000 rpm for 10 min at 4°C. Sodium dodecyl sulfate (SDS) sample buffer was added to the cleared lysate and boiled for 4 min before separation by SDS-polyacrylamide gel electrophoresis. Variability in cell densities was minimized by normalization to total protein concentration measured with the protein assay kit (Bio-Rad). Proteins were blotted to polyvinylidene difluoride membranes (Bio-Rad), and blots were blocked in 5% milk in TBS + 0.1% Tween and horseradish peroxidase-conjugated goat anti-rabbit IgG (1:20000; A0545, Sigma) as a second antibody. Immunoreactive proteins were detected using a chemiluminescence kit (Biological Industries) according to the manufacturer’s protocol.

Whole mount antibody staining

Whole mount antibody staining was performed as described previously (Shive et al., 2000). Embryos were incubated with anti-human ADHFe1 rabbit polyclonal antibody (1:500; PA5-31416; Thermo Fisher Scientific) and alkaline phosphatase-conjugated goat anti-rabbit IgG (1:2000; A0545, Sigma) as a secondary antibody. The localization was visualized in BM-Purple solution (Roche). When needed, we performed a second staining using the FITC molecules using an anti-FITC antibody (1:10000; Roche) and stained using BICP.

Cell culture and transfection

HEK293 cells were grown in DMEM supplemented with 10% fetal calf serum, at 37°C and 5% CO2 atmosphere. For transfection, cells were plated on a 6-well plate, grown to 50% confluence and transfected using polyethyleneimine (PEI; Sigma). Briefly, 2 μg of DNA were used per well mixed with PEI to a ratio of 1:5 respectively. Cells were co-transfected with 1 μg of each plasmid (pCS2-AdhFe1 and pCS2-GFP). Transfection efficiency of transiently transfected cells was routinely 70% or more estimated by microscopy. After 48 hours, cells were harvested and lysed for qPCR or Western blot analysis.

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