Hypoxia controls Flvcr1 gene expression in Caco2 cells through HIF2α and ETS1

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ABSTRACT

The tissue-specific gene expression changes mediated by the hypoxia inducible factors (HIFs) allow the adaptation of cells to low oxygen tension and control several processes including erythropoiesis, angiogenesis and vasculogenesis. The Feline Leukemia Virus, subgroup C, Receptor 1 (Flvcr1) gene encodes for two isoforms, FLVCR1a and 1b, involved in the export of heme out of the cell and of mitochondria respectively. Studies in mouse models demonstrated a crucial role of FLVCR1 isoforms in erythropoiesis and during embryo development.

Here, we showed the modulation of Flvcr1 gene expression in different tissues and cell lines in response to hypoxia. Chromatin immunoprecipitation analysis demonstrated that HIF2α and HIF-dependent transcription factor Ets1 (v-ets avian erythroblastosis virus E26 oncogene homolog 1) bind at the region -318/+39 of the Flvcr1 promoter. Analysis of Caco2 cells in which Hif2α or Ets1 were silenced or overexpressed demonstrated that, both HIF2α and ETS1 are involved in the transcriptional regulation of Flvcr1a and that HIF2α is absolutely required for Flvcr1a induction upon hypoxia.

The inclusion of the Flvcr1 gene in the group of HIF2α-responsive genes strengthens its role in hypoxia-stimulated processes like erythropoiesis, vasculogenesis and heme absorption.

KEY WORDS

Hypoxia, transcriptional regulation, Flvcr1, HIF2α, ETS1.

ABBREVIATIONS

HIF, hypoxia inducible factor; Flvcr1, Feline Leukemia Virus, subgroup C, Receptor 1; ETS1, v-ets avian erythroblastosis virus E26 oncogene homolog 1; VEGFR2, vascular endothelial growth factor receptor-2; VEGF, vascular endothelial growth factor; FPN1, Ferroportin 1; DMT1, Divalent Metal Transporter 1; DcytB, Duodenal Cytochrome B; ActD, actinomycin D; L-Mim, L-mimosine; HRE, hypoxia responsive element; qRT-PCR, quantitative real-time polymerase chain reaction; shRNA, short hairpin RNA; ChIP, chromatin immunoprecipitation.

1. INTRODUCTION

During hypoxia the key transcription regulatory proteins hypoxia inducible factors (HIF) modulate the expression of genes involved in the response to low oxygen levels by binding the hypoxia

responsive elements (HRE) present in their promoters [1]. Hypoxia responsive genes include genes involved in iron metabolism, erythropoiesis, angiogenesis and development.

The Feline Leukemia Virus, subgroup C, Receptor 1 (Flvcr1) gene encodes for two isoforms: FLVCR1a, a plasma membrane heme exporter mediating heme export out of the cell [2, 3], and FLVCR1b, located on the mitochondrion and regulating heme trafficking between mitochondria and cytosol [4] .
Flvcr1 is expressed ubiquitously[5] and it has a crucial role in erythropoiesis and during embryo development in mice[2, 3].

Here we show that Flvcr1 gene is regulated by hypoxia through HIF2α and the HIF-regulated transcription factor ETS1.

2. MATERIALS AND METHODS

2.1. RNA extraction and quantitative real-time PCR analysis

Total RNA extraction and quantitative real-time PCR (qRT-PCR) were performed as previously reported [6].

2.2. Cell culture

The human colorectal adenocarcinoma Caco2 cells (ATCC: HTB-37™) were propagated in appropriate standard conditions. Hypoxia was achieved by incubation in a hypoxia chamber (Billups-Rothenberg Inc., Del Mar CA) containing 1% oxygen, 5% carbon dioxide and 94% nitrogen gas or mimicked by treatment with 500 μM L-mimosine (Sigma-Aldrich, Milano, Italy) for 24 hours in serum-free medium.
Actinomycin D was from Sigma-Aldrich, Milano, Italy.

2.3. Hif2α and Ets1 silencing

For gene silencing, short hairpin RNA (shRNAs) used were all from TRC Lentiviral (pLKO.1) Human shRNA set (Thermo Fisher Scientific, Inc. Waltham MA, USA). Following lentiviral transduction, cells were selected with 0.02μg/ml puromycin.

2.4. Cloning procedures

The 5′ region -318/+39 flanking human Flvcr1a first exon was subcloned into pSTBlue-1 vector (pSTBlue-1 Perfectly Blunt Cloning Kit, EMD Millipore Novagen, Billerica Ma, USA) and then into the pGL3-basic luciferase reporter vector (Promega Corporation, Madison WI, USA).

2.5. Transient transfection and luciferase assay

For Hif-2α over-expression, pcDNA3–Hif2α was provided by David Russell (University of Texas Southwestern Medical Center, Dallas, Texas, USA). Analyses were performed 24 hours following transient transfection.

For luciferase assay the pRL-TK *Renilla* luciferase normalizing vector (Promega Corporation, Madison WI, USA) was cotransfected with luciferase constructs at a 1:10 ratio. pGL3-basic or pGL3–Flvcr1a vectors (500 ng) were cotransfected with pcDNA3, pcDNA3–Hif-2α, pCMV-SPORT6 or pCMV-SPORT6-Ets1 expression vectors (250ng). pCMV-SPORT6 and pCMV-SPORT6-Ets1 were by Thermo Fisher Scientific, Inc. (Waltham MA, USA, Clone ID 3676286).

The assay was performed using a Dual-Glo Luciferase Reporter Kit on a Glomax Multi Detection System (all by Promega Corporation, Madison WI, USA) 48 hours following transfection.

2.6. ChIP assay

Cells were maintained in hypoxia for 24 hours or incubated with 500 μM L-Mimosine (Sigma-Aldrich, Milano, Italy) in serum-free medium for 24 hours.

The ChIP assay was performed according to a protocol previously described [7]. Anti–HIF-2α (NB100-122; Novus Biologicals) and anti-ETS1 (N-276 sc-111, Santa Cruz Biotechnology,Inc CA, USA) antibodies were used for immunoprecipitation. Oligonucleotide sequences used for qRT-PCR are indicated in supplemental data.

2.7. Statistical Analysis

Results were expressed as mean ± SEM. Statistical analyses were performed using one-way or two-way analysis of variance followed by the Bonferroni correction for multiple group comparisons. An unpaired Student's t-test was used when only two groups were compared. A P value of less than 0.05 was regarded as significant.

3. RESULTS

3.1. Flvcr1 expression is regulated in hypoxia.

In order to study the regulation of Flvcr1 gene in oxygen restricted conditions, Flvcr1a and Flvcr1b mRNA levels were analysed upon hypoxia in Caco2 cells.

The comparison between the relative amount of Flvcr1a and Flvcr1b transcripts in Caco2 cells showed higher mRNA levels for the 1a isoform (Fig. S1). Interestingly, an increase of both Flvcr1a and Flvcr1b mRNA levels was observed 24 hours upon hypoxia (Fig. 1A) or following the treatment with a known hypoxia-mimicking agent, L-mimosine (Fig. 1B). The hypoxia-dependent increase of Flvcr1a and 1b was completely prevented by actinomycin D, indicating that transcriptional processes are responsible for their up-regulation during hypoxia (Fig. 1C). The hypoxic modulation of Flvcr1a and 1b mRNA levels was confirmed in additional cell lines and in tissues from wild-type mice maintained in hypoxia (Fig. S2).

To get insight into the transcriptional regulation of Flvcr1 during hypoxia, the genomic regions flanking the first and the second exon of the human Flvcr1 gene, where the transcription start points of Flvcr1a and 1b isoforms are located, were analysed. Interestingly, putative HRE sequences very close to putative ETS1 candidate binding sites were observed around the transcriptional start site of the Flvcr1a isoform, suggesting the involvement of ETS1 and HIF transcription factors in the control, at least, of the Flvcr1a isoform.

ETS1 is a known HIF2α partner transcriptionally activated by HIF1α [8] and that act together with HIF subunits in the regulation of many target genes [9, 10]. As expected, Ets1 mRNA transcriptionally increased following hypoxia (Fig. S3A, B) and L-mimosine treatment (Fig. S3C) in Caco2 cells.

Thus, these data indicate that Flvcr1 transcription is modulated in hypoxia and point to HIFs and ETS1 as the candidate transcription factors involved in this process. Although HIF1α and HIF3α involvement in the regulation of Flvcr1 can not be excluded, subsequent analyses were focused on the HIF2α subunit, as it was demonstrated that HIF2α is the subunit mostly involved in the control of iron genes in intestinal cell-like Caco2 cells [11].

3.2. The modulation of Flvcr1a expression upon hypoxia is controlled by HIF2α and ETS1.

The involvement of HIF2α and ETS1 in the hypoxic regulation of Flvcr1a was investigated.

Chromatin immunoprecipitation assays confirmed the binding of both HIF2α and ETS1 to the Flvcr1a promoter (Fig. 2A, B and S4). Interestingly, while hypoxia was required for HIF2α binding, a tendency to ETS1 binding was already observed in normoxia, and the contact was enhanced by hypoxia.

To better characterize the direct binding of these two transcription factors to the Flvcr1 promoter, the fragment -318/+39 of the Flvcr1 promoter was cloned into a luciferase reporter vector, this

region containing a putative HRE sequence located at -75/-68, very close to three candidate ETS1 binding sites (Fig. 2C). We observed a significant induction of the luciferase activity in Caco2 cells over-expressing HIF2α or ETS1 (Fig. 2D, E).

Finally, the need of both HIF2α and ETS1 for the hypoxic induction of Flvcr1a was investigated. HIF2α over-expression in Caco2 cells increased Flvcr1a mRNA levels (Fig. 2F), while HIF2α-depleted cells (Fig. S5A) failed to show the hypoxia-dependent increase of the Flvcr1a transcript (Fig. 2G). Interestingly, HIF2α-depleted cells showed a strong increase of Ets1 mRNA level (Fig. 2H). Together, these data indicate that HIF2α is crucial for the hypoxic regulation of Flvcr1a and that ETS1 alone is not sufficient to induce Flvcr1a transcription upon hypoxia.

Also ETS1-depleted cells (Fig. S5B) failed to up-regulate Flvcr1a mRNA following hypoxia (Fig. 2I). Nevertheless, Hif2α mRNA levels were significantly lower in these cells upon hypoxia as compared with control cells (Fig. 2J), so it was not possible to exclude that the lack of Flvcr1a induction in ETS1-depleted cells could depend on HIF2α insufficiency.

Collectively, these data demonstrate that both HIF2α and ETS1 are involved in the transcriptional regulation of Flvcr1a and, particularly, that HIF2α is absolutely required for Flvcr1a induction upon hypoxia.

4. DISCUSSION

The data reported herein show that the Flvcr1 gene is transcriptionally regulated upon hypoxia and that, in Caco2 cells, this regulation is mediated by HIF2α and ETS1 transcription factors.

The present work focused on the binding of HIF2α to the Flvcr1a promoter in Caco2 cells. However, it can not be excluded that hypoxia could regulate the Flvcr1 gene through different HIF subunits in other cell types. Indeed, the regulation of gene expression by HIFs is complex and it is known that different tissues could favour the expression of a preferential HIF subunit [6]. Moreover, distinct HIF subunits could regulate different genes [12], but it is also possible that two different HIF subunits could generate opposite effects on the same gene in a certain tissue [13]. Finally, the same HIF subunit can exert opposite effects on the same gene depending on the cell type [14]. The differential hypoxic regulation of Flvcr1 in various tissues and cell lines further supports these considerations, suggesting that Flvcr1 expression could be unaffected by oxygen depletion in certain tissues like the spleen, or up-regulated in other tissues like the duodenum and the muscle, or down-regulated in some cell lines like Mel cell line.

A strong collaboration between HIF2α and ETS1 emerged by the analysis of the Flvcr1a hypoxic modulation in Caco2 cells. The reported results show a complex regulation existing between HIF2α and ETS1. ETS1 expression is known to be induced by many transcription factors [15], including HIF1α [8]. In the present work a strong increase of Ets1 mRNA level appeared following depletion of HIF2α in Caco2 cells. Moreover, the down-regulation of ETS1 was associated to a decrease in Hif2α mRNA. Although the reason for these modulations is unknown, they suggest the existence of a complex interaction between these two transcription factors, perhaps more tricky than that described so far.

Apart the reciprocal regulation of ETS1 and HIF2α, the results indicate that both HIF2α and ETS1 are involved in the hypoxic modulation of Flvcr1a, as their binding to the Flvcr1a promoter is enhanced in low oxygen tension conditions. Moreover, the data demonstrate that HIF2α is absolutely required for the hypoxic transcriptional induction of Flvcr1a. Previous studies have stressed the importance of cooperation between ETS1 and HIF2α in the modulation of many hypoxia-regulated genes [9], particularly genes that are crucial for vascular development like the vascular endothelial growth factor receptor-2 (Vegfr2) [10]. Moreover, HIFs, together with factors of the ETS family, have been shown to play critical roles during embryo development for angiogenesis and vasculogenesis [16]. Vascular development is a coordinated process during which endothelial precursor cells arise from the mesoderm, proliferate, migrate, aggregate and form a vascular network. These processes require changes in cell proliferation, adhesion, motility, and cell spreading that are controlled by genes directly activated by HIFs and ETS family transcription factors [17, 18]. ETS targets include matrix degrading proteases, cell adhesion molecules and receptor tyrosine kinases for the angiopoietin and vascular endothelial growth factor (VEGF)-mediated signaling pathways, all of which have essential roles in the vasculature [17, 18]. Interestingly, Flvcr1a-null mice die at midgestation due to vascular abnormalities [4]. Therefore, Flvcr1a could be part of the network regulated by hypoxia during proper vascular development.

Different hypotheses could be proposed to explain the increase of Flvcr1 mRNA levels in intestinal cells upon hypoxia both in vitro and in vivo. First, it can be supposed that FLVCR1a acts as a heme exporter in intestinal cells. In this case, the up-regulation of Flvcr1a in response to hypoxia could be crucial to support heme-iron delivery to the erythropoietic compartments in a moment of high iron demand, thus complementing the supply of inorganic iron mediated by the intestinal iron exporter Ferroportin 1 (FPN1). Another hypothesis is that FLVCR1-mediated heme export from mitochondria and cytosol could be crucial to limit local cell oxidative stress in the intestinal mucosa

during hypoxia. Indeed, studies have demonstrated that hypoxia is a triggering stimulus for redox disturbances at distinct levels of cellular organization [19]. HIF2α mediates the transcriptional regulation of several "anti-oxidant genes", as indicated by increased oxidative stress and reactive oxygen species dependent-tissue damage in Hif2α-null mice [20]. Moreover, it has been demonstrated a pivotal role for HIF2α in the regulation of many iron-related genes in the duodenum, particularly the Divalent metal transporter 1 (Dmt1), the Duodenal cytochrome B (DcytB) and Fpn1 [11, 21, 22]. Thus, during hypoxia HIF2α regulates the expression of both "iron absorption-genes" and "anti-oxidant genes". This likely favours iron absorption to meet enhanced erythropoietic demand associated to hypoxia, and concomitantly counteracts iron-dependent oxidative stress in a moment of high iron trafficking in enterocytes. In this context FLVCR1 (1a and 1b) could likely take part to the anti-oxidant response to hypoxia, that involves mitochondrial block, heme export out of mitochondria, and consequent cellular heme detoxification, the last two steps likely mediated by FLVCR1b and FLVCR1a respectively.

Finally, our findings have important implications for erythropoiesis. Indeed, the physiologic response to hypoxia is the increase in red blood cell production. HIFs orchestrate this response by inducing specific gene expression changes that result in increased erythropoietin production by the kidney, increased iron absorption and utilization and in changes in bone marrow microenvironment that facilitate erythroid progenitors proliferation and maturation [23]. Flvcr1 gene plays a crucial role in erythroid differentiation to ensure adequate heme supply for hemoglobinization [2, 4]. The data reported herein indicate that Flvcr1 is part of the HIF-mediated response to hypoxia, a function that is consistent with its role in erythropoiesis. Surprisingly, the erythroid cell lines Mel and K562 respond to hypoxia by down-regulating Flvcr1a and 1b or Flvcr1a alone, respectively, a quite unexpected response for a gene involved in erythroid differentiation. Nevertheless, the physiologic response of erythroid progenitors to hypoxia is mainly mediated by the activation of the erythropoietin receptor and involves the activation of a complex differentiation program aimed at increasing iron uptake, heme synthesis and globin expression. Thus, it is likely that the finely regulated expression of Flvcr1 isoforms during the differentiation of erythroid progenitors derives from the integration of different signals among which those mediated by HIF and Ets families.

5. CONCLUSIONS

In conclusion, the data reported in the present work demonstrate the involvement of HIF2α and ETS1 transcription factors in the regulation of the Flvcr1a isoform in Caco2 cells. Furthermore,

they lead to the inclusion of Flvcr1 in the group of HIF responsive genes, strengthening its role in hypoxia-stimulated processes like erythropoiesis, vasculogenesis and likely heme absorption.

AUTHORSHIP AND DISCLOSURES

VF designed and performed the experiments, made the analyses and wrote the paper; FN performed ChIP experiments; VP performed some experiments, LS, FA and SO were involved in study design and discussed the data; ET designed the experiments, analysed data and wrote the paper. The authors declare that no competing interests exist.

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FIGURE LEGENDS

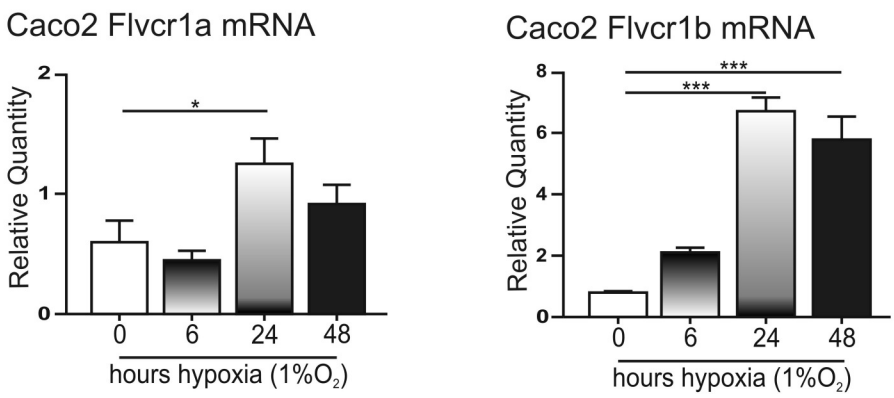
Fig. 1. Hypoxia controls Flvcr1 gene expression. (A) qRT-PCR analysis of Flvcr1a and Flvcr1b expression in Caco2 cells following 6, 24 and 48 hours of hypoxia (1% oxygen). Transcript abundance, normalized to βActin RNA expression, is expressed as a fold increase over a calibrator sample. Data represent mean ± SEM, n=5; *=P<0.05, ***=P<0.001. (B) qRT-PCR analysis of Flvcr1a and Flvcr1b expression in Caco2 cells following treatment with the hypoxia-mimicking agent L-Mimosine (L-Mim) for 24 hours. Transcript abundance, normalized to βActin RNA expression, is expressed as a fold increase over a calibrator sample. Data represent mean ± SEM, n=3; *=P<0.05. (C) qRT-PCR analysis of Flvcr1a and Flvcr1b expression in Caco2 cells treated with increasing amount of actinomycin D (ActD) and maintained 24 hours in hypoxia (1% oxygen). Transcript abundance, normalized to βActin RNA expression, is expressed as a fold increase over a calibrator sample. Data represent mean ± SEM, n=3; **=P<0.01, ***=P<0.001.

Fig. 2. The modulation of Flvcr1a expression upon hypoxia is controlled by HIF-2α and ETS1. (A, B) ChIP analysis of an extended region of the Flvcr1a promoter using a HIF2α antibody (A) or an ETS1 antibody (B). As a negative control, the binding to an Flvcr1a promoter region very distant respect to the transcriptional start site was analysed. The experiment was performed in Caco2 cells maintained 24 hours in hypoxia (1% oxygen) or treated with the hypoxia-mimicking agent L-mimosine (L-Mim) for 24 hours. Data represent mean ± SEM, n=3; *=P<0.05, **=P<0.01, ***=P<0.001. Results shown are representative of two independent experiments. (C) Schematic diagram of Flvcr1 promoter illustrating the putative ETS1 and HIF binding sites in the regulatory region upstream the transcription start point of the Flvcr1a isoform. (D, E) Analysis of luciferase activity in Caco2 cells transiently cotransfected with reporter constructs containing the Flvcr1a promoter region -318/+39 cloned upstream of the luciferase coding sequence together with a empty pcDNA3 vector or a pcDNA3 vector expressing HIF2α (D) or with a empty pCMVSport6 vector or a pCMVSport6 vector expressing ETS1 (E). Relative luciferase activity, normalized to renilla luminescence, is expressed as a fold increase over a calibrator sample. Data represent mean ± SEM, n=3; ***=P<0.001. Results shown are representative of three independent experiments. (F) qRT-PCR analysis of Flvcr1a expression in Caco2 cells over-expressing HIF2α. Transcript abundance, normalized to βActin RNA expression, is expressed as a fold increase over a calibrator sample. Data represent mean ± SEM, n=3; *=P<0.05. (G) qRT-PCR analysis of Flvcr1a expression in Caco2 cells maintained in hypoxia (1% oxygen) for 24 hours and in which the expression of HIF2α was down-regulated using a specific shRNA. Transcript abundance, normalized to βActin RNA

expression, is expressed as a fold increase over a calibrator sample. Data represent mean ± SEM, n=5; ***=P<0.001. (H) qRT-PCR analysis of Ets1 expression in Caco2 cells maintained in hypoxia (1% oxygen) for 24 hours and in which the expression of HIF2α was down-regulated using a specific shRNA. Transcript abundance, normalized to βActin RNA expression, is expressed as a fold increase over a calibrator sample. Data represent mean ± SEM, n=5; ***=P<0.001. (I) qRT-PCR analysis of Flvcr1a expression in Caco2 cells maintained in hypoxia (1% oxygen) for 24 hours and in which the expression of ETS1 was down-regulated using a specific shRNA. Transcript abundance, normalized to βActin RNA expression, is expressed as a fold increase over a calibrator sample. Data represent mean ± SEM, n=4; **=P<0.01. (J) qRT-PCR analysis of Hif2α expression in Caco2 cells maintained in hypoxia (1% oxygen) for 24 hours and in which the expression of ETS1 was down-regulated using a specific shRNA. Transcript abundance, normalized to βActin RNA expression, is expressed as a fold increase over a calibrator sample. Data represent mean ± SEM, n=5; **=P<0.01, ***=P<0.001.

Figure 1

A


Caco2 Flvcr1a mRNA
Relative Quantity
0
1
2
*
0
6
24
48
hours hypoxia (1%O2)
Caco2 Flvcr1b mRNA
Relative Quantity
0
2
4
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0
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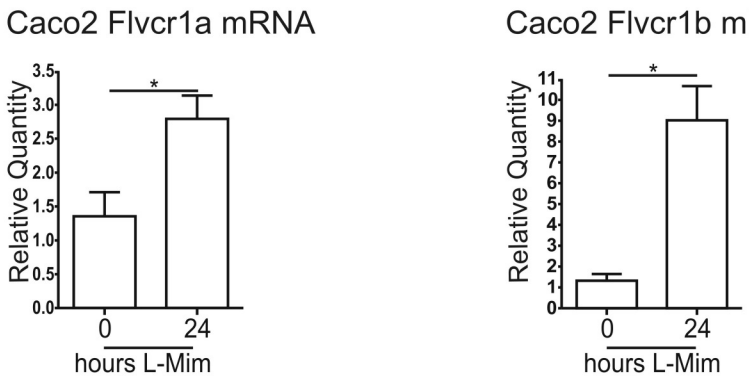
24
48
hours hypoxia (1%O2)

B


Caco2 Flvcr1a mRNA
Relative Quantity
0.0
0.5
1.0
1.5
2.0
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3.0
3.5
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24
hours L-Mim
Caco2 Flvcr1b mRNA
Relative Quantity
0
1
2
3
4
5
6
7
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9
10
11
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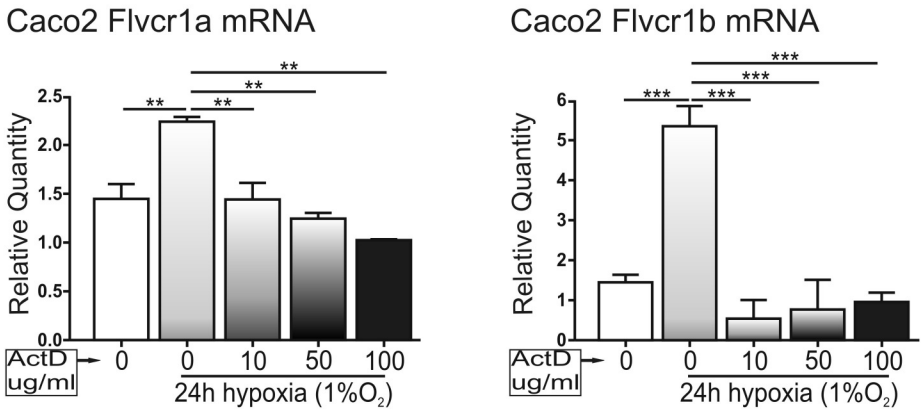
hours L-Mim

C


Caco2 Flvcr1a mRNA
Relative Quantity
0.0
0.5
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1.5
2.0
2.5
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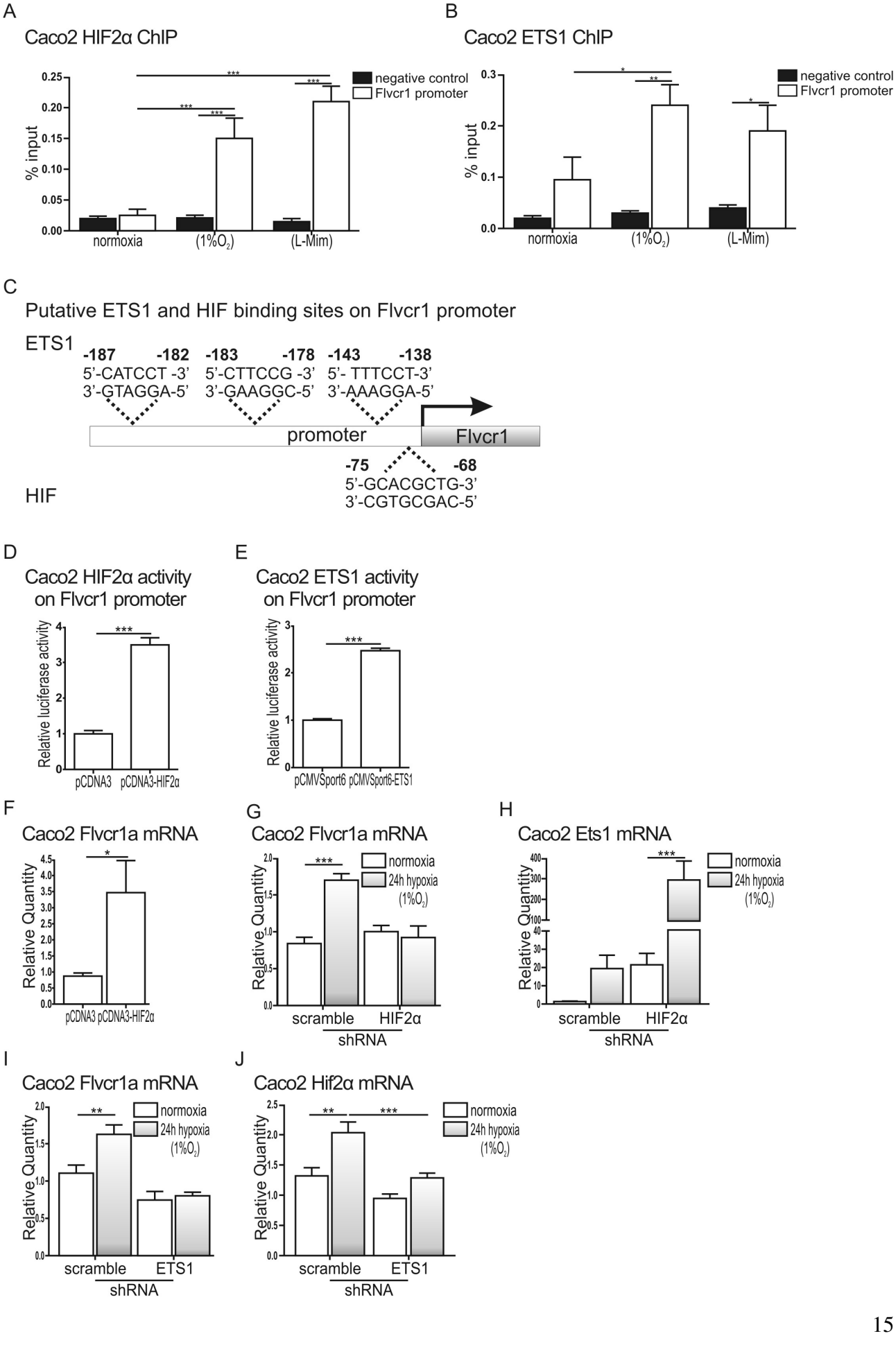
ActD
ug/ml
0
0
10
50
100
24h hypoxia (1%O2)
Caco2 Flvcr1b mRNA
Relative Quantity
0
1
2
3
4
5
6
***
***
***
***
ActD
ug/ml
0
0
10
50
100
24h hypoxia (1%O2)

Figure 2


A
Caco2 HIF2α ChIP
negative control
Flvcr1 promoter
% input
normoxia
(1%O2)
(L-Mim)
B
Caco2 ETS1 ChIP
C
Putative ETS1 and HIF binding sites on Flvcr1 promoter
ETS1
-187 -182
5'-CATCCT -3'
3'-GTAGGA-5'
-183 -178
5'-CTTCCG -3'
3'-GAAGGC-5'
-143 -138
5'- TTTCCT-3'
3'-AAAGGA-5'
promoter
Flvcr1
-75 -68
5'-GCACGCTG-3'
3'-CGTGCGAC-5'
HIF
D
Caco2 HIF2α activity on Flvcr1 promoter
Relative luciferase activity
pCDNA3
pCDNA3-HIF2α
E
Caco2 ETS1 activity on Flvcr1 promoter
pCMVSport6
pCMVSport6-ETS1
F
Caco2 Flvcr1a mRNA
Relative Quantity
G
Caco2 Flvcr1a mRNA
normoxia
24h hypoxia
(1%O2)
scramble
HIF2α
shRNA
H
Caco2 Ets1 mRNA
I
Caco2 Flvcr1a mRNA
ETS1
J
Caco2 Hif2α mRNA

SUPPLEMENTS

SUPPLEMENTAL METHODS

Animals

Two month-old wild-type male mice in the Sv129 background were used. Mice were placed in a hypoxia chamber (Invivo$_2$ 200 Gas Mixer Q, Ruskinn) at a temperature of 25°C for 24 hours. Hypoxia was achieved by decreasing oxygen level to 8% and adjusting nitrogen level to 92%. Carbon dioxide was 0.03%. Controls were left at room air.

All experiments were approved by the animal studies committee of the University of Torino (Italy).

Cell culture

The human lung carcinoma A549 cell line (ATCC number CCL-185TM), the murine monocyte/macrophage Raw 264.7 cell line (ATCC number TIB-71), the murine fibroblast NIH/3T3 cell line (ATCC number CRL16-58), the human K-562 cell line (ATCC number CCL-243) and the murine erythroleukemia Mel cells, kindly provided by Sonia Levi (Life and Health San Raffaele University, Milan, Italy), were propagated in appropriate standard conditions. Mel differentiation was obtained adding 1.8% dimethyl sulfoxide in the medium since 24 hours before the hypoxia experiment.

SUPPLEMENTAL TABLE

Table S1. Sequences of primers used for Chromatin immunoprecipitation (ChIP) and cloning.

Primers used for ChIP		
name	Forward 5'-3'	Reverse 5'-3'
Flvcr1a-chip	GCCTCCAATTCTTCCCTG	AGCTACCGGGCGAGAACT
Flvcr1a-negative-chip	AGGCCTCACTCTGTTGCCTA	GTTCAAGACCAGCCTGGGTA
HRE3-DMT1-1A-chip	CTCTGATGTCTGCTCGTGGA	TGAAACCATTAACCCCAAGC

Primers used for FLVCR1a cloning for luciferase assay		
name	Forward 5'-3'	Reverse 5'-3'
Flvcr1a-luciferase-cloning	GCCTCCAATTCTTCCCTG	CCCAAGCTTCGTGAACAGATGAAGGTCTCC

ChIP primers Flvcr1a-chip amplify the promoter region -318/-106 preceding the transcriptional start site of Flvcr1a. ChIP primers Flvcr1a-negative-chip amplify a region -5249/-5056 preceding the transcriptional start site of Flvcr1a and were used as a negative control. ChIP primers HRE3-DMT1-1A-chip amplify the HRE3 region of DMT1-1A [1] and were used as a positive control.
Cloning primers Flvcr1a-luciferase-cloning amplify the region -318/+39 respect to the Flvcr1a transcriptional start site. Reverse primer contain XhoI restriction site sequence (underlined).

SUPPLEMENTAL FIGURES

Figure S1
Caco2 Flvcr1a and 1b mRNA

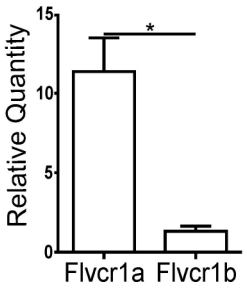


Fig. S1. Higher Flvcr1a mRNA levels as compared with Flvcr1b mRNA in Caco2 cells. qRT-PCR analysis of Flvcr1a and Flvcr1b expression in Caco2 cells in normoxia. Transcript abundance, normalized to βActin RNA expression, is expressed as a fold increase over a calibrator sample. Data represent mean ± SEM, n=3; *=P<0.05.

Figure S2

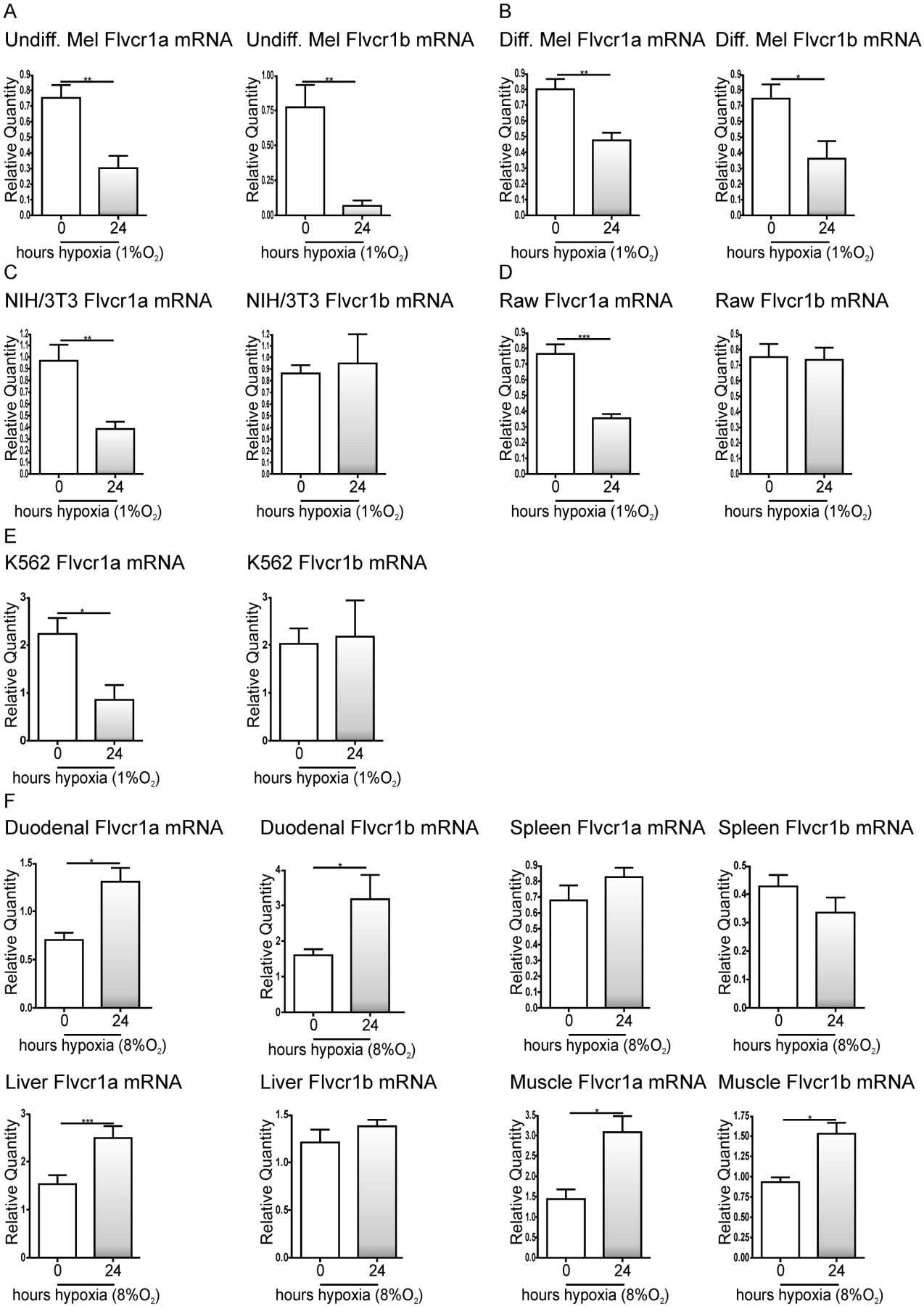

A
Undiff. Mel Flvcr1a mRNA
Undiff. Mel Flvcr1b mRNA
B
Diff. Mel Flvcr1a mRNA
Diff. Mel Flvcr1b mRNA
C
NIH/3T3 Flvcr1a mRNA
NIH/3T3 Flvcr1b mRNA
D
Raw Flvcr1a mRNA
Raw Flvcr1b mRNA
E
K562 Flvcr1a mRNA
K562 Flvcr1b mRNA
F
Duodenal Flvcr1a mRNA
Duodenal Flvcr1b mRNA
Spleen Flvcr1a mRNA
Spleen Flvcr1b mRNA
Liver Flvcr1a mRNA
Liver Flvcr1b mRNA
Muscle Flvcr1a mRNA
Muscle Flvcr1b mRNA
Relative Quantity
hours hypoxia (1%O2)
hours hypoxia (8%O2)

Fig. S2. Flvcr1 gene expression is modulated upon hypoxia in cells and mouse tissues. (A, B) qRT-PCR analysis of Flvcr1a and Flvcr1b expression in undifferentiated (A) or differentiated (B) Mel cells following 24 hours of hypoxia (1% oxygen). Transcript abundance, normalized to 18S RNA expression, is expressed as a fold increase over a calibrator sample. Data represent mean ± SEM, n=6; *=P<0.05, **=P<0.01. (C) qRT-PCR analysis of Flvcr1a and Flvcr1b expression in NIH/3T3 cells following 24 hours of hypoxia (1% oxygen). Transcript abundance, normalized to 18S RNA expression, is expressed as a fold increase over a calibrator sample. Data represent mean ± SEM, n=5; **=P<0.01. (D) qRT-PCR analysis of Flvcr1a and Flvcr1b expression in Raw 264.7 cells following 24 hours of hypoxia (1% oxygen). Transcript abundance, normalized to 18S RNA expression, is expressed as a fold increase over a calibrator sample. Data represent mean ± SEM, n=5; ***=P<0.001. (E) qRT-PCR analysis of Flvcr1a and Flvcr1b expression in K-562 cells following 24 hours of hypoxia (1% oxygen). Transcript abundance, normalized to βActin RNA expression, is expressed as a fold increase over a calibrator sample. Data represent mean ± SEM, n=5; *=P<0.05. (F) qRT-PCR analysis of Flvcr1a and Flvcr1b expression in the duodenum, spleen, liver and muscle of wild-type mice following 24 hours of exposure to hypoxia (8% oxygen). Transcript abundance, normalized to 18S RNA expression, is expressed as a fold increase over a calibrator sample. Data represent mean ± SEM, n=4; *=P<0.05, ***=P<0.001.

Figure S3

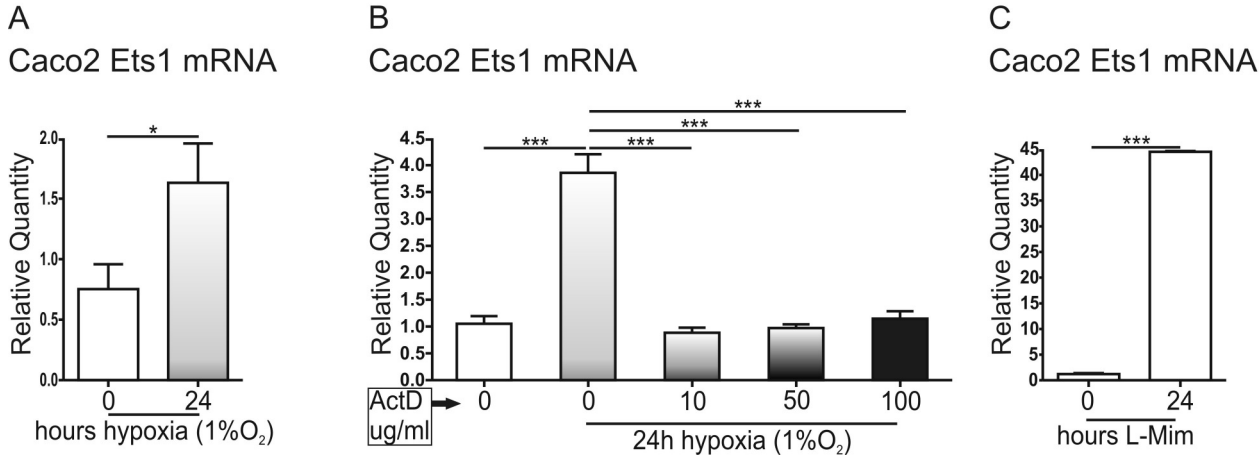


Fig. S3. ETS1 gene expression is increased by hypoxia. (A) qRT-PCR analysis of Ets1 expression in Caco2 cells maintained in hypoxia (1% oxygen) for 24 hours. Transcript abundance, normalized to βActin RNA expression, is expressed as a fold increase over a calibrator sample. Data represent mean ± SEM, n=4; *=P<0.05. (B) qRT-PCR analysis of Ets1 expression in Caco2 cells treated with increasing amount of actinomycin D (ActD) and maintained 24 hours in hypoxia (1% oxygen). Transcript abundance, normalized to βActin RNA expression, is expressed as a fold increase over a calibrator sample. Data represent mean ± SEM, n=3; ***=P<0.001. (C) qRT-PCR analysis of Ets1 expression in Caco2 cells following treatment with the hypoxia-mimicking agent L-Mimosine (L-Mim) for 24 hours. Transcript abundance, normalized to βActin RNA expression, is expressed as a fold increase over a calibrator sample. Data represent mean ± SEM, n=3; ***=P<0.001.

Figure S4

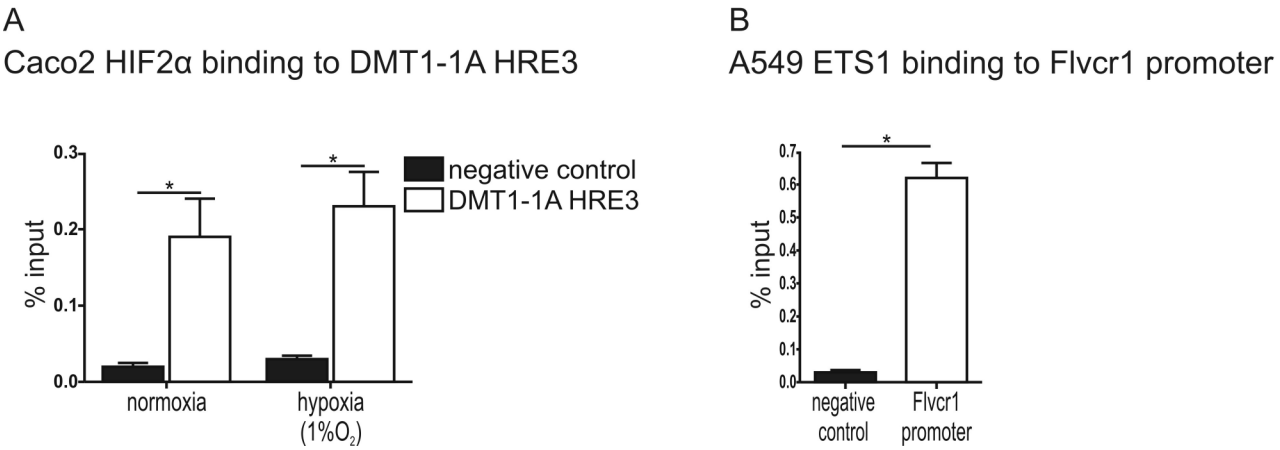


Fig. S4. ChIP assay positive controls. As a positive control for ChIP assay, HIF2α binding to the DMT1-1A hypoxia responsive element 3 (HRE3) promoter region [1] was checked. A further positive control was provided by the analysis of ETS1 binding to Flvcr1a promoter in A549 cells, as reported in ENCODE Project Database [2]. (A) ChIP analysis of the DMT1-1A promoter region HRE3 using a HIF2α antibody. The experiment was performed in Caco2 cells maintained 24 hours in hypoxia (1% oxygen). Data represent mean ± SEM, n=3; *=P<0.05. Results shown are representative of two independent experiments. (B) ChIP analysis of an extended region of the Flvcr1a promoter using an ETS1 antibody. As a negative control, the binding to an Flvcr1a promoter region very distant respect to the transcriptional start site was analysed. The experiment was performed in A549 cells in normoxia. Data represent mean ± SEM, n=3; *=P<0.05. Results shown are representative of two independent experiments.

Figure S5

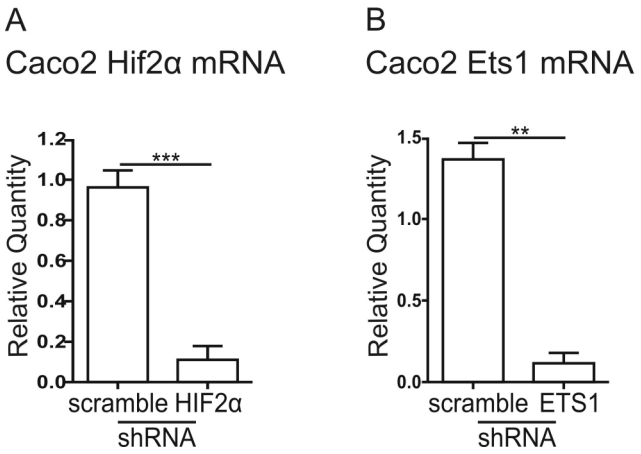


Fig. S5. ShRNAs-mediated HIF2α and ETS1 down-regulation. (A) qRT-PCR analysis of Hif2α expression in Caco2 cells in normoxia upon HIF2α down-regulation using a specific shRNA. Transcript abundance, normalized to βActin RNA expression, is expressed as a fold increase over a calibrator sample. Data represent mean ± SEM, n=6; ***=P<0.001. (B) qRT-PCR analysis of Ets1 expression in Caco2 cells in normoxia upon ETS1 down-regulation using a specific shRNA. Transcript abundance, normalized to βActin RNA expression, is expressed as a fold increase over a calibrator sample. Data represent mean ± SEM, n=3; **=P<0.01.

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