Siderophore-mediated iron trafficking in humans is regulated by iron

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Supplementary Figure legends

Supplementary Figure 1. Immunoblot analysis of hBDH2 in naïve or DFO treated MCF-10 A cells. Cell lysates were resolved on discontinuous polyacrylamide gels and blots were probed with anti-human BDH2 antibody, anti-Ferritin antibody (positive control), and anti-Actin antibody (loading control).

Supplementary Figure 2. Mouse *bdh2* expression is not regulated by iron. Mouse *Tfr1* and *bdh2* mRNAs were quantified by real time PCR analysis 16 hours post treatment in murine kidney cells (MIMCD) or pro-B lymphocytic cells (FL5.12). The value on *y*-axis was set at 1.0 for the mRNA levels in untreated cells. The relative mRNA levels in each sample were normalized to *actin* mRNA. Positive control includes *Tfr1*, whose expression is affected by iron. Results shown are the average means of three independent experiments with error bars depicting SD. Indicated *p* values in comparison with mock treatment.

Supplementary Figure 3. *hBDH2* IRE confers iron-dependent regulation onto a heterologous reporter gene. (A) Schematic diagram of the Luciferase reporter plasmids. **(B)** Secondary structures of wt and mutant *hBDH2* IREs. Mutated nucleotides are shaded. **(C)** MCF-10 A cells were transfected with Luciferase reporter plasmids shown in (A) for 5 hours and subsequently treated with 100 μM each FAC or DFO overnight before harvesting for the determination of the Luciferase activity. Luciferase light units are normalized using the Renilla Luciferase activity to account for transfection efficiency. All experiments were performed in triplicate, and error bars represent the SD. Indicated *p* values were in comparison with mock treatment.

Supplementary Figure 4. RNAi knockdown of IRPs. (**A**) HeLa cells were initially transfected with an empty vector (control) or vectors expressing shRNAs specific for IRPs. G418 resistant clones were in turn transfected with a Dharmacon SMARTpool siRNAs specific for IRP-1 and IRP-2 or a scrambled siRNA oligomer (control). The mRNA levels of *IRPs* were assessed by qRT-PCR with gene-specific primers. The value on *y*-axis was set at 1.0 for the mRNA levels in control cells. The relative mRNA levels in each sample were normalized to *actin* mRNA. Results shown are the average means of three independent experiments with error bars depicting SD; $*p < 0.05$ in comparison with control cells. (**B & C**) Deficiency of IRPs leads to abrogation of iron-dependent

regulation of *hBDH2* mRNA. HeLa cells stably expressing shRNAs targeting IRP-1 or IRP-2 were in turn transfected with indicated siRNA oligomers to achieve a complete knock down of IRPs. *TfR1* and *hBDH2* mRNAs were quantified by real time PCR analysis 16 hours after Hemin or DFO treatment (100 μM each). The value on *y*-axis was set at 1.0 for the mRNA levels in untreated cells. The relative mRNA levels in each sample were normalized to *actin* mRNA. Positive control includes *TfR1*, whose expression is affected by iron. Results shown are the average means of three independent experiments with error bars depicting SD.

Supplementary Figure 5. Requirement of *hBDH2* IRE for iron-dependent regulation. MCF-10 A cells stably expressing control shRNAs (**A & B**) or shRNAs targeting IRP-1 and-2 (**C & D**). IRP-1 or IRP-2 shRNA expressing cells were in turn transiently transfected with IRP-1 or IRP-2 siRNA pools to achieve complete knockdown of IRPs. Control cells or cells depleted of IRPs were treated with 100 μM each of hemin or DFO overnight, and then Actinomycin D was at 5 μg/ml added to inhibit the transcription. *hBDH2* and *TfR1* mRNAs were quantified by real time PCR analysis at indicated time points. The relative mRNA levels in each sample were normalized to *rlp13a* then plotted against time on a semi logarithmic graph. The first order decay slope was calculated using linear regression (Graph Pad Prism 5). The value on *y*-axis represents the percentage *hBDH2* or *TfR1* mRNA remaining relative to time point zero. Results shown are a representative of three independent experiments. **(E)** Murine FL5.12 cells were treated with 100 μM each of hemin or DFO overnight, and then Actinomycin D was at 5 μg/ml added to inhibit the transcription. *mbdh2* and *Tfr1* mRNAs were quantified by real time PCR analysis as described above. **(F)** Expression levels of IRP-1 and IRP-2 in MCF-10 A cells stably expressing control shRNAs or shRNAs as well as siRNA pools targeting IRP-1 and IRP-2 were analyzed by an immunoblot.

Supplementary Figure 6. *hBDH2* IRE binds to IRPs *in vitro*. (**A**) Radiolabeled RNA probes corresponding to IREs of *hBDH2* or *TfR1 B IRE* were incubated with increasing amounts of purified recombinant IRP-1 or IRP-2. The RNA:protein complexes were resolved in a polyacrylamide gel and visualized by autoradiography. (**B**) The specificity of interaction between *hBDH2* IRE and IRPs was assessed using a radiolabeled *hBDH2* wt IRE

probe and an increasing molar excess unlabeled competitor WT, MT-1, MT-2, or TfR1 B IRE RNAs. F, indicates free RNA probe.

Table S1. Sequences for RT-PCR Primers

RT-PCR primers	Sequence (5' to 3')	Notes
Luc2	TGCAAAAGATCCTCAACGTG	Forward
	AATGGGAAGTCACGAAGGTG	Reverse
hRluc	ACAAGTACCTCACCGCTTGG	Forward
	GACACTCTCAGCATGGACGA	Reverse
mbdh2	GTTGCTGGTTTTGTCCACCACGGAAC	Forward
	GTCTGTTTAGGAAAGTTTTCAGTGCCTC	Reverse
mactb	TGTTACCAACTGGGACGACA	Forward
	GGGGTGTTGAAGGTCTCAAA	Reverse
mtfr1	GTTTCTGCCAGCCCCTTATTAT	Forward
	GCAAGGAAAGGATATGCAGCA	Reverse
hBDH2	ACCTGATGATCAAGGCATTC	Forward
	TCACAACTCCTTTGACGCTG	Reverse
hACTb	CAGCCATGTACGTTGCTATCCAGG	Forward
	AGGTCCAGACGCAGGATGGCATG	Reverse
hTfR1	AAAATCCGGTGTAGGCACAG	Forward
	CCTTTAAATGCAGGGACGAA	Reverse
18srRNA	CGGCTACCACATCCAAGGAA	Forward
	GCTGGAATTACCGCGGCT	Reverse
rlp13a	CTCAAGGTCGTGCGTCTG	Forward
	TGGCTTTCTCTTTCCTCTTCTC	Reverse
eif2b2	TCAAGATTATCCGGGAGGAG	Forward
	ATGGAAGCTGAAATCCTCGT	Reverse
pbdh2-1	ACCTGATGATCAAGGCATTC	Forward for Rhesus and Baboon
	TCACAACTCCTTTGATGCTG	Reverse
pbdh2-2	ACCTGATGATCAAGGCATTC	Forward for Tamarin and Marmoset
	TCACAACTCCTTTAATGCTG	Reverse
ptfr1-1	AAAATCCAGTGTGGGGACAG	Forward for Rhesus and Baboon
	CCTTTAAATGCAAGGATGAA	Reverse
ptfr1-2	AAAGTCCAGTGTCGGAACAG	Forward for Tamarin and Marmoset
	CCTTTAAATGCAAGGACGAA	Reverse

Table S2. Hemochromatosis samples described in Figs 1 A and B*

Order	Age	Gender	Genotype	Iron	IBC	TF saturation (%)	Ferritin μg/L
1	52	Female	C282 Het	153	264	58	56.7
2	52	"	"	132	337	39	51.1
3	65	"	"	78	315	25	30.3
4	55	Male	"	69	307	22	123.5
5	47	Female	"	134	308	44	60.6
6	69	Male	"	86	245	35	169.4
7	32	Female	"	88	333	26	107.9
8	54	"	"	116	311	37	158.5
9	54	"	"	N/D	N/D	34.7	1048
10	35	Male	C282Y Homo	106	284	37	499.7
11	40	"	"	106	349	30	111.9
12	33	Female	"	70	406	17	52.4
13	40	"	"	88	282	31	77.4
14	43	"	"	145	379	38	108
15	39	"	"	69	379	18	88.9
16	57	"	"	51	269	19	261.6
17	54	Male	"	N/D	N/D	99.7	1234
18	53	Female	Compound Het	88	255	35	51.7
19	27	"	"	51	344	15	65.3
20	35	"	"	51	324	16	84.6
21	41	"	"	41	370	11	51.4
22	33	"	"	59	366	16	26.4
23	41	"	"	67	309	22	260.1
24	29	"	"	75	395	19	18.9
25	53	Male	"	N/D	N/D	N/D	724
26	57	Female	H63D Het	60	356	17	104.5
27	35	"	"	N/D	N/D	N/D	33.2
28	42	Male	"	113	266	42	220.1
29	55	Female	"	46	349	13	34.4
30	61	"	"	40	303	13	55.7
31	43	"	"	75	281	27	88.4
32	54	"	"	43	319	13	43.9
33	41	Male	"	93	394	24	102.9
34	35	Female	"	74	331	22	74.1
35	47	"	"	57	412	14	13.7
36	72	"	Normal	85	384	22	73.5
37	41	"	"	49	337	15	49.3
38	48	"	"	71	343	21	41
39	46	"	"	76	273	28	52.3
40	44	"	"	85	357	24	16.2

*RNA was extracted from flash frozen biopsies from normal and HH livers. Iron is serum iron level. IBC, iron binding capacity.

Table S3. Hemochromatosis samples described in Fig 1 C*

Order	Age	Gender	Genotype	Hepatic Iron index	Iron	TF saturation (%)	Ferritin µg/L
1	50	Male	C282 Homo	5.35	151	97	3425
2	66	Female	"	4.32	194	84	574
3	51	Male	"	4.66	N/D	90	1030.3
4	38	"	C282Y Het	2.35	N/D	74	423.5
5	42	"	"	2.1	98	74	460.6
6	59	Male	Normal	1.2	85	25	109.2
7	42	Female	"	1	39	26	89.9
8	54	"	"	1.3	51	27	58.5
9	44	Male	"	1.12	N/D	24.7	108.8

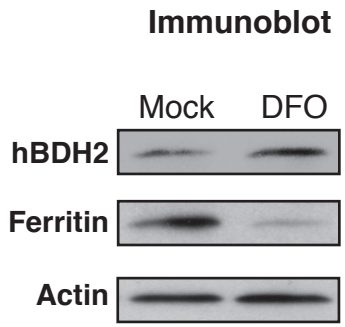
*Specimens were obtained from liver biopsies in case of normal and for HH sections were obtained from liver explants. Iron is serum iron level.

Table S4. Relative levels of *hBDH2* and *TfR1* in microarray analysis of hemochromatosis samples*

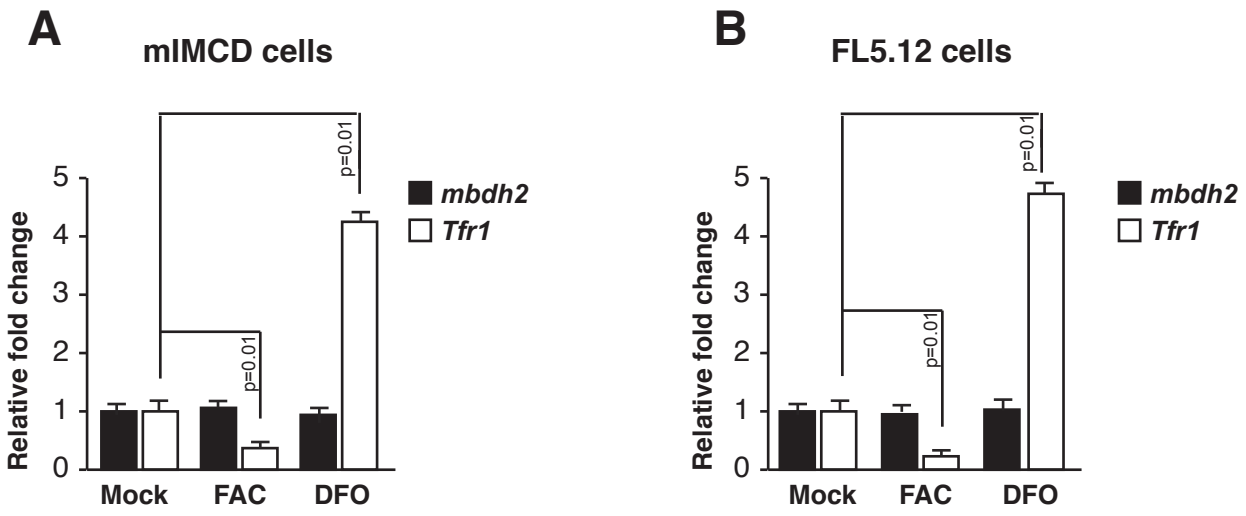
Probe	Normal1	Normal2	Normal3	HH1	HH2	HH3
hBDH2	0.97	0.17	0.66	-0.55	-0.67	-0.82
***TfR1* probe 1**	2.01	0.62	1.09	1.26	1.17	0.54
***TfR1* probe 2**	0.36	0.02	0.24	-0.19	-0.66	0.28
***TfR1* probe 3**	-0.96	-0.60	-1.76	-0.80	-0.95	-0.32

*Microarray data was obtained from ref. 39.

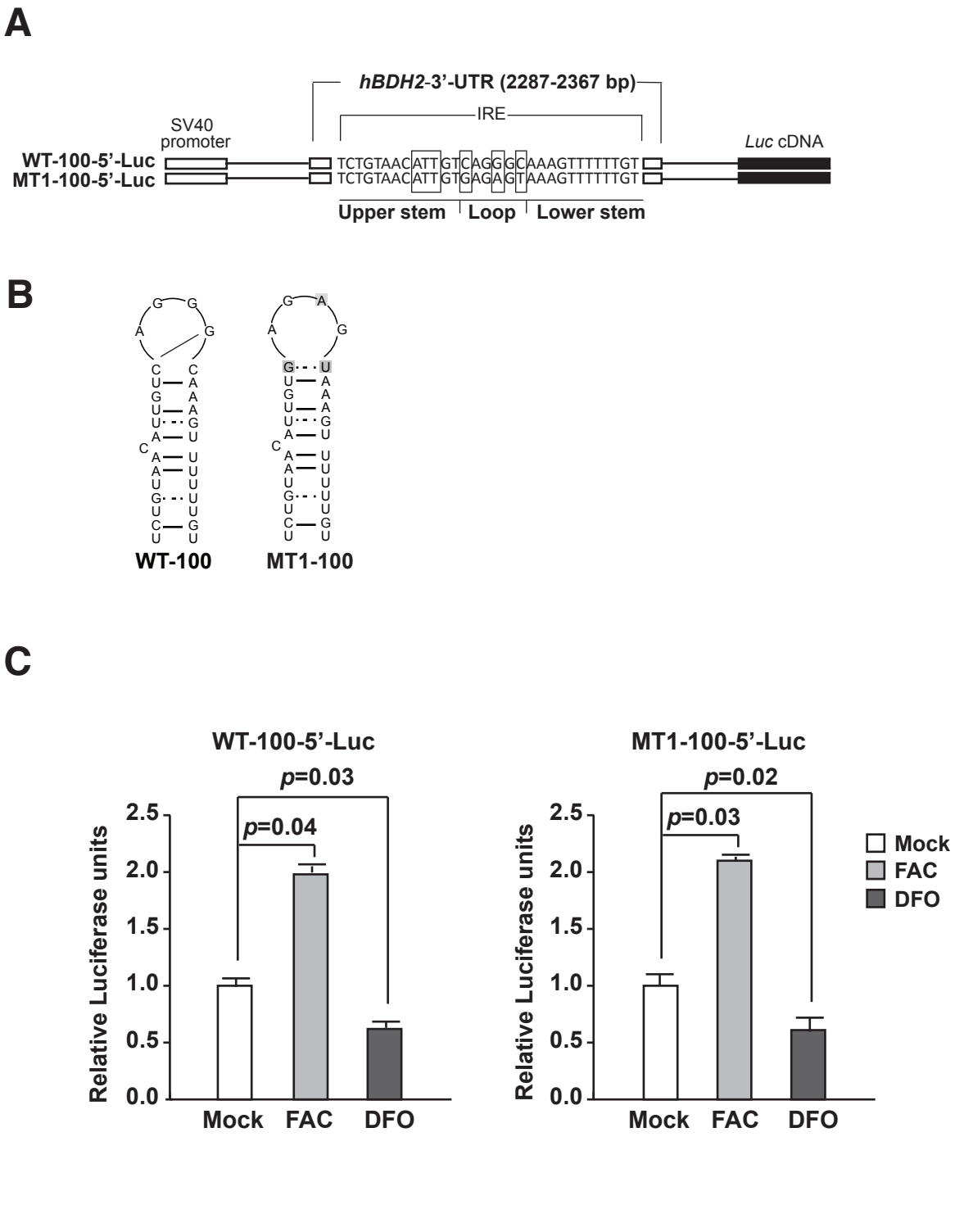
Supplementary Figure 1



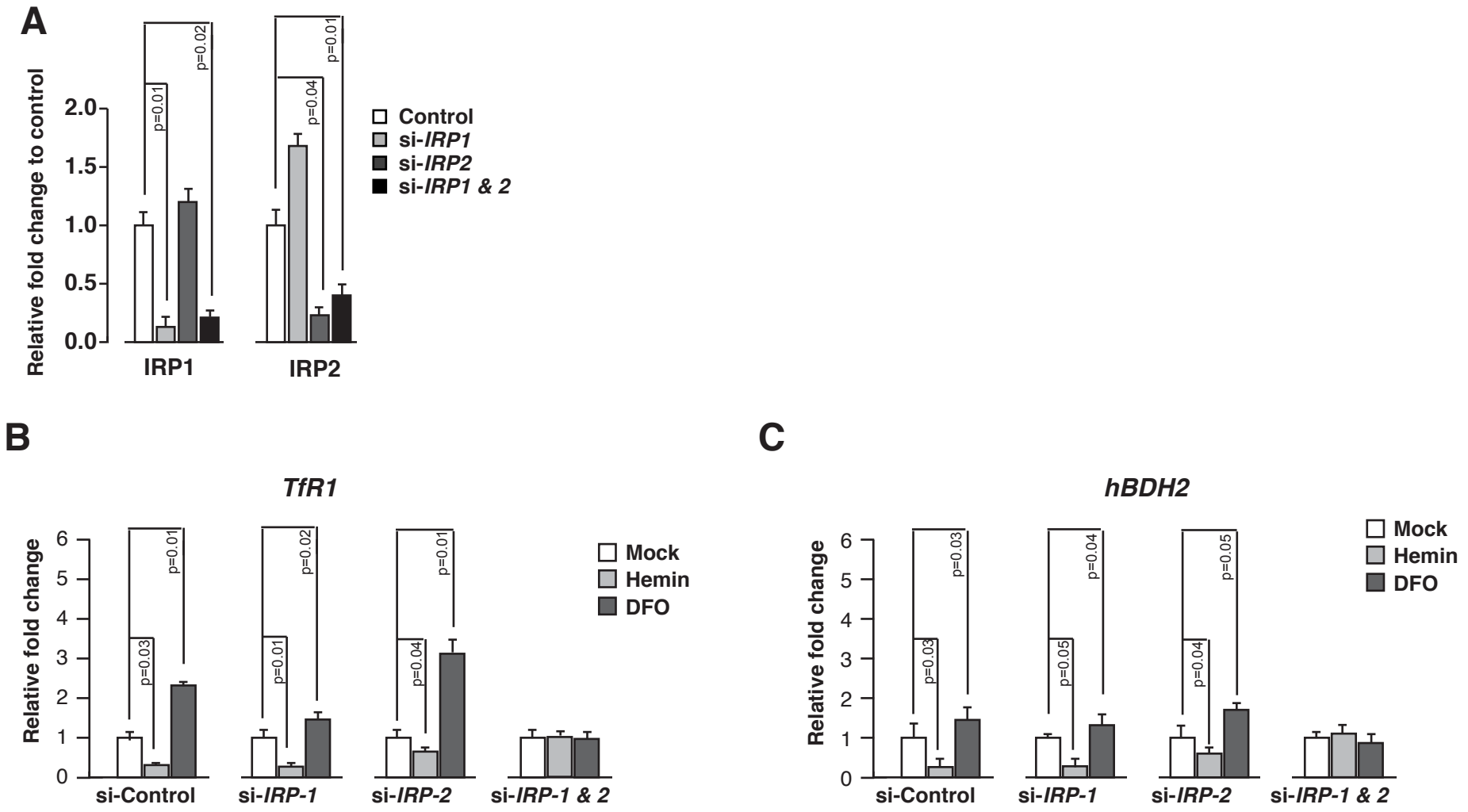
Supplementary Figure 2



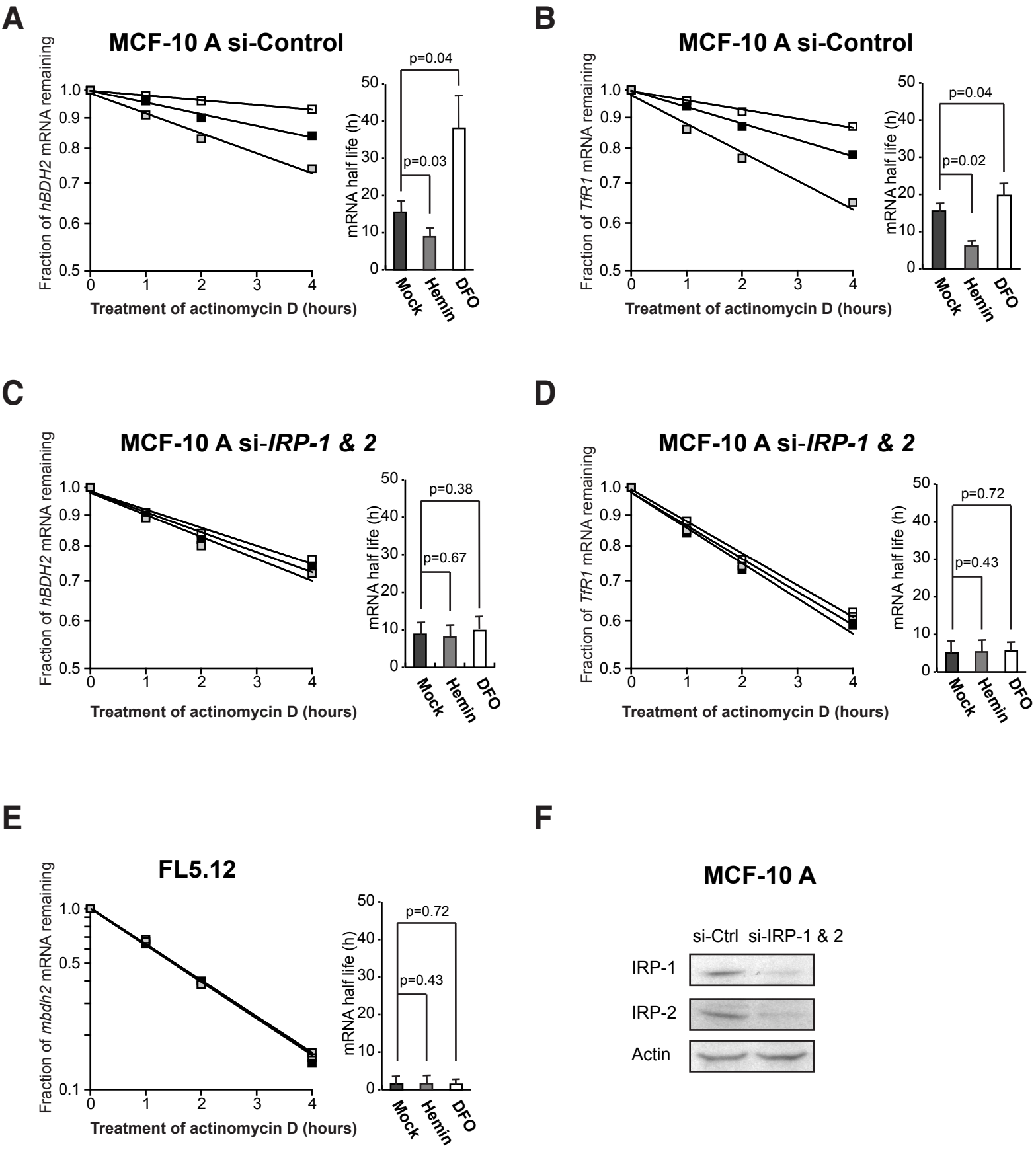
Supplementary Figure 3



Supplementary Figure 4



Supplementary Figure 5



Supplementary Figure 6