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Iron Chelator-Mediated Alterations in Gene Expression: Identification of Novel Iron-Regulated Molecules That Are Molecular Targets of Hypoxia-Inducible Factor-1α and p53

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ABSTRACT

Iron deficiency affects 500 million people, yet the molecular role of iron in gene expression remains poorly characterized. In addition, the alterations in global gene expression after iron chelation remain unclear and are important to assess for understanding the molecular pathology of iron deficiency and the biological effects of chelators. Considering this, we assessed the effect on whole genome gene expression of two iron chelators (desferrioxamine and 2-hydroxy-1-napthylaldehyde isonicotinoyl hydrazone) that have markedly different permeability properties. Sixteen genes were significantly regulated by both ligands, whereas a further 50 genes were significantly regulated by either compound. Apart from iron-mediated regulation of expression via hypoxia inducible factor-1α, it was noteworthy that the transcription factor p53 was also involved in iron-regulated gene expression. Examining 16 genes regulated by both chelators in normal and neoplastic cells, five genes (*APP*, *GDF15*, *CITED2*, *EGR1*, and *PNRC1*) were significantly differentially expressed between the cell types. In view of their functions in tumor suppression, proliferation, and apoptosis, these findings are important for understanding the selective antiproliferative effects of chelators against neoplastic cells. Most of the genes identified have not been described previously to be iron-regulated and are important for understanding the molecular and cellular effects of iron depletion.

Iron deficiency affects approximately 500 million people. However, despite the enormity of this problem, very little is understood concerning the precise molecular roles played by iron in growth, cell-cycle progression, and apoptosis.

Iron plays essential roles in cells, including DNA synthesis and cell cycle control (Buss et al., 2003). It is well known that iron deficiency induced by chelators results in a G_1/S arrest and apoptosis (Buss et al., 2003). The best-characterized role of iron in proliferation involves its function in the rate-limiting step of DNA synthesis catalyzed by ribonucleotide reductase (Buss et al., 2003). Iron has also been shown to regulate the expression of a variety of molecules involved in cell-cycle control (e.g., $p21^{CIP1/WAF1}$, GADD45, p53, cyclin D1, etc.) (Gao and Richardson, 2001; Liang and Richardson, 2003) and metastasis suppression (e.g., *N*-myc downstream-regulated gene-1; NDRG-1) (Le and Richardson, 2004). Because iron is important for mitochondrial heme and iron sulfur cluster synthesis, it is likely that iron chelation will also affect basic mitochondrial metabolism and function.

Iron chelators are well known therapeutics for the treatment of iron-overload disease and some of these agents show potential for cancer therapy (Buss et al., 2003; Whitnall et al., 2006). In general, lipophilic chelators are more effective at inhibiting [^{3}H]thymidine incorporation, DNA synthesis, and proliferation than their hydrophilic counterparts (Rich-

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ABBREVIATIONS: 311, 2-hydroxy-1-napthylaldehyde isonicotinoyl hydrazone; DFO, desferrioxamine; IRP, iron-regulatory protein; IRE, iron-responsive element; UTR, untranslated region; HIF-1, hypoxia-inducible factor-1; HUVEC, human umbilical vein endothelial cell; MEF, murine embryonic fibroblast; Tet, tetracycline; RT, reverse transcription; PCR, polymerase chain reaction; Dp44mT, di-2-pyridylketone 4,4-dimethyl-3-thiosemicarbazone; APP, amyloid β (A4) precursor protein; GDF15, growth differentiation factor 15; FAC, ferric ammonium citrate; CON, control medium alone; TfR1, transferrin receptor 1; BNIP3, BCL2/adenovirus E1B 19-kDa interacting protein 3; CITED2, Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxyl-terminal domain, 2; EGR1, early growth response 1; ERO1L, ERO1-like; GDF15, growth differentiation factor 15; NDRG-1, *N*-myc downstream regulated gene 1; PNRC1, proline-rich nuclear receptor coactivator 1; PPM1D, protein phosphatase 1D magnesium-dependent, 32 δ isoform.

ardson et al., 1995). A good example of this is provided by comparing the activity of the tridentate lipophilic ligand 2-hydroxy-1-napthylaldehyde isonicotinoyl hydrazone (311; Fig. 1A) and the hexadentate hydrophilic compound desferrioxamine (DFO; Fig. 1A). Both are high-affinity iron(III) chelators (Richardson and Bernhardt, 1999) that demonstrate distinct differences in activity (Richardson et al., 1994, 1995; Darnell and Richardson, 1999). In fact, DFO shows limited permeability and iron chelation efficacy, which leads to low antiproliferative efficacy, whereas 311 is membrane-permeable and demonstrates marked antiproliferative effects (Richardson et al., 1994, 1995; Darnell and Richardson, 1999).

Unlike other cytotoxic chelators, upon binding iron, DFO and 311 do not generate cytotoxic radicals, their effects being due to induction of iron depletion (Chaston et al., 2003). In addition, DFO and 311 up-regulate the iron-regulated gene *transferrin receptor 1* (*TfR1*) (Darnell and Richardson, 1999).

Important regulators of intracellular iron status are the iron-regulatory proteins 1 and 2 (IRP1 and -2) that bind conserved iron-responsive elements (IREs) in the 3′- and 5′-untranslated regions (UTRs) of mRNAs that play roles in iron metabolism (Sanchez et al., 2007). Previous studies showed that DFO and 311 deplete cellular iron and effectively increase IRP-RNA-binding activity (Darnell and Richardson, 1999). Depending on iron status, the IRPs post-transcriptionally regulate the expression of genes, including *TfR1*, which is involved in iron uptake, and *ferritin H-* and *L-chain*, which play crucial roles in iron storage (Sanchez et al., 2007). However, this is not the only mechanism controlling gene expression in response to iron; the other well known system is mediated by hypoxia-inducible factor-1α (HIF-1α) (An et al., 1998; Semenza, 1999). The expression of the transcription factor p53 can also be regulated by iron (An et al., 1998; Liang and Richardson, 2003). It is noteworthy that HIF1-α is thought to stabilize p53 and lead to its up-regulation after iron depletion (An et al., 1998).

HIF-1 is activated under hypoxia and/or iron depletion and is composed of two subunits, a constitutively expressed β-subunit and the α-subunit (Semenza, 1999). Un-

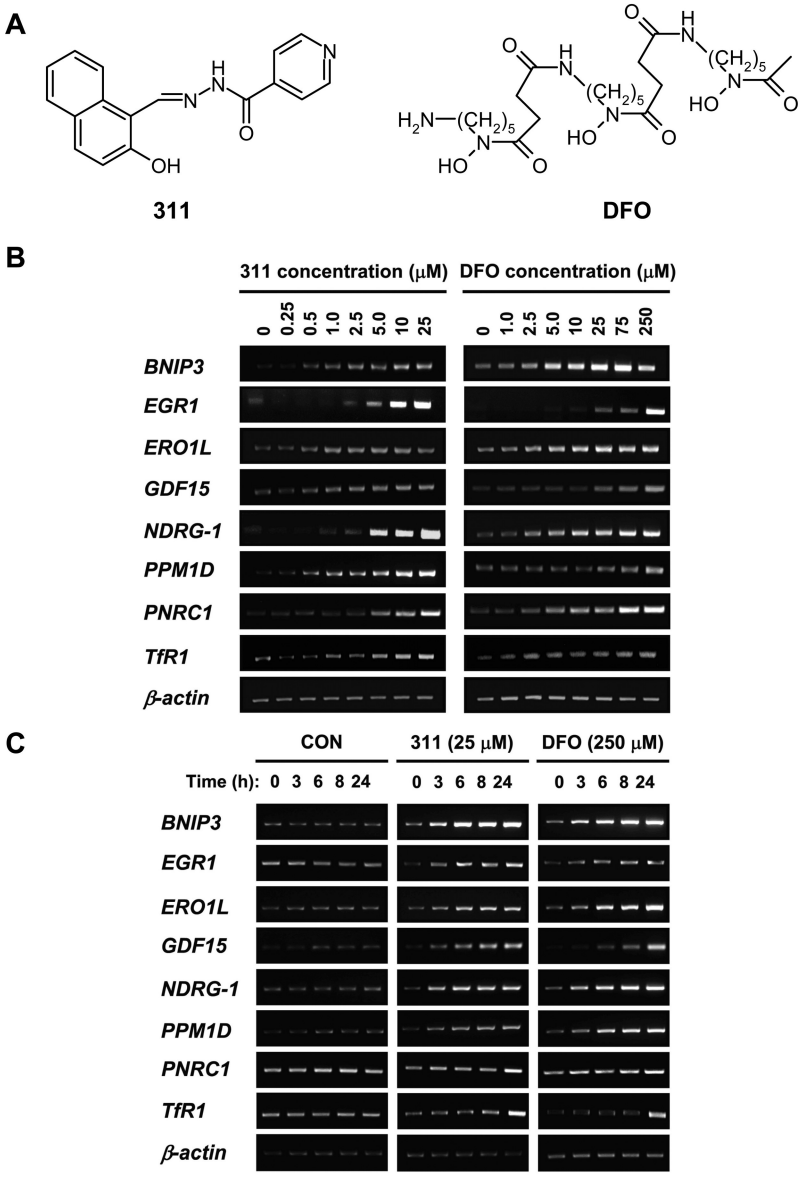

Fig. 1. A, structures of the chelators DFO and 311. B, the effect of various concentrations of 311 (0.25–25 μM) or DFO (0.1–250 μM) after a 24-h incubation at 37°C on the mRNA expression of seven genes commonly up-regulated by either of these ligands (see Table 2) in MCF-7 breast cancer cells. C, the effect of incubation time (3–24 h at 37°C) with 311 (25 μM) or DFO (250 μM) on the mRNA expression of the seven genes in B in MCF-7 cells. In B and C, *TfR1* expression has been assessed as a positive control for iron depletion. Results in B and C are representative photographs of gels from three separate experiments.

der normal oxygen tension and iron levels, HIF-1α is regulated by prolyl hydroxylase, which allows binding to the von Hippel-Lindau protein. This protein activates ubiquitin E3 ligase, resulting in HIF-1α degradation via the proteasome (Semenza, 1999). Under oxygen and/or iron depletion, prolyl hydroxylase fails to function, leading to HIF-1α accumulation and nuclear translocation, where it binds to HIF-1β to form the HIF-1 complex, which regulates genes, such as the *TfR1* (Bianchi et al., 1999), by binding to hypoxia response elements (Semenza, 1999).

In this investigation, we examined the effect of iron chelation on global gene expression after incubation of cells with either DFO or 311. These studies were initiated to achieve a more comprehensive understanding of the cellular response to iron depletion, which remains only preliminary. We identified a range of iron-regulated genes that play roles in diverse biological processes, including tumor suppression, proliferation, and apoptosis. Furthermore, this work was designed to also investigate further potential reasons for the selective antiproliferative activity of iron chelators in neoplastic relative to normal cells. Five iron-regulated genes have been identified that were regulated differently in neoplastic cells than in normal cells and could play a role in the selective antitumor effects of chelators.

Materials and Methods

Chelators

311 was synthesized and characterized as reported (Richardson and Bernhardt, 1999). DFO was from Novartis (Basel, Switzerland).

Cell Culture

Human MCF-7 breast cancer cells, MRC-5 fibroblasts, SK-Mel-28 melanoma cells, DMS53 lung carcinoma cells, and SK-N-MC neuroepithelioma cells were from the American Type Culture Collection (Manassas, VA) and cultured as described previously (Le and Richardson, 2004). Human fibroblasts were from Corielle (Camden, NJ) and grown under the same conditions as the cells above. Primary cultures of human umbilical vein endothelial cells (HUVECs) were a gift from Mr. Pat Pisansarakit (Heart Research Institute, Sydney, Australia). The MCF-7 and MRC-5 cells were used in initial experiments as their response to iron chelation by DFO and 311 have been well studied (Yuan et al., 2004). Murine embryonic fibroblasts (MEFs) from control and homozygous *HIF-1α* knockout mice were obtained from Dr. R. Johnson (University of California, San Diego). H1299 cells (*p53*-null) stably transfected with tetracycline (Tet)-regulated p53 (Stein et al., 2004) were obtained from GenHunter (Nashville, TN).

Microarray Processing

The MCF-7 cells were incubated with either control medium (CON; minimum essential medium containing 10% fetal calf serum) or control medium containing DFO (250 μM) or 311 (25 μM) for 24 h at 37°C. These concentrations and this incubation period were used because we demonstrated that under these conditions, the chelators up-regulate iron-responsive genes (e.g., *TfR1*) (Darnell and Richardson, 1999). Moreover, the higher concentration of DFO was implemented because of its limited ability to permeate membranes (Richardson et al., 1994; Darnell and Richardson, 1999). Total RNA was isolated from cells in 1 ml of TRIzol reagent (Invitrogen, Sydney, Australia). First-strand cDNA synthesis was performed using 15 μg of RNA by the Affymetrix One Cycle cDNA synthesis kit, and the cDNA products were purified via the Affymetrix GeneChip sample clean-up kit (Affymetrix, Santa Clara, CA). Using the Affymetrix in vitro transcription labeling kit, biotin-labeled cRNA was prepared from the cDNA.

After purification with the above-mentioned clean-up kit, the quantity of the products was ascertained using the Nanochip protocol on a Bioanalyser 2100 (Agilent Technologies, Santa Clara, CA). Labeled cRNA (20 μg) was fragmented to 50 to 200 base pairs, and then its quality was checked again. Samples (cRNA, 0.05 μg/μl) that passed this checkpoint were then prepared for hybridization to the Human Genome U133 Plus 430 2.0 array. This consists of >47,000 transcripts from over 38,500 genes. On completion of hybridization and washing, microarray chips were scanned with the Affymetrix GeneChip Scanner 3000 (Affymetrix).

Microarray Data Analysis

Gene Expression Analysis. A two phase strategy was used to identify differentially expressed genes. First, genome-wide screening was performed using Affymetrix GeneChips. The empirical Bayes procedure (Suryo Rahmanto et al., 2007) was applied to detect genes most likely to be differentially expressed between control and chelator-treated samples. Individual p values were then adjusted using the Holm step-down procedure to reduce false positives (Suryo Rahmanto et al., 2007). Statistical analyses of data from Affymetrix Genechips were used to produce a list of genes with $p < 0.05$. This analysis was not meant to provide proof of differential expression. Rather, definitive evidence of differential expression was obtained from RT-PCR assessment of samples used for the microarray and at least three other independent samples.

Annotation. Functional annotation of genes was assigned through Gene Ontology (http://www.geneontology.org) and classifications obtained through the public databases NetAffx (http://www.affymetrix.com/analysis/index.affx) and DAVID (http://david.abcc.ncifcrf.gov) (Suryo Rahmanto et al., 2007).

Data Availability. The complete data set can be accessed on the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) using accession numbers GSM444834, GSM444835, and GSM444836.

RNA Isolation, RT-PCR, and Western Analysis

Total RNA was isolated using TRIzol (Invitrogen) (Suryo Rahmanto et al., 2007). Semiquantitative RT-PCR was performed using the SuperScript III RT/Platinum Taq Mix (Suryo Rahmanto et al., 2007). RT-PCR was shown to be semiquantitative by an optimization protocol that demonstrated it was in the log-phase of amplification. The sequences of the primers implemented are listed in Table 1. The housekeeping gene, *β-actin*, was coamplified as an internal standard. Semiquantitative RT-PCR was used as it provides a sensitive, reproducible, and rapid estimation of gene expression and was more economical than quantitative real-time PCR. Protein isolation and Western analysis were performed using established techniques (Gao and Richardson, 2001).

Statistical Analysis

Results are expressed as mean ± S.D. All experiments were performed at least three times. Excluding the statistical analyses of the microarray results, all data were compared using the Student's t test. Data were considered statistically significant when $p < 0.05$.

Results

Little is known concerning global changes in gene expression after iron depletion using chelators. To address this, we performed whole genome gene array using MCF-7 breast cancer cells. This was necessary as previous studies using other methods only examined preselected genes that have known roles or putative functions in iron metabolism (Muckenthaler et al., 2003). Assessment of a whole genome array is vital for identifying genes regulated by iron and for understanding the response to iron depletion. Demonstration of

these alterations in expression was also essential in elucidating the effects of iron chelation on cells at the molecular level and to understand why some ligands show selective anticancer activity (Buss et al., 2003; Whitnall et al., 2006).

In our studies, we compared DFO and 311, which show low and high membrane permeability, respectively (Richardson et al., 1994). Furthermore, 311 shows greater antiproliferative activity than DFO and is far more effective at inducing iron mobilization from normal and neoplastic cells (Richardson et al., 1995). Hence, it was important to compare the difference in response at the molecular level for both compounds. Whereas other chelators, such as di-2-pyridylketone 4,4-dimethyl-3-thiosemicarbazone (Dp44mT), have been designed that show far greater antiproliferative activity than DFO or 311 (Yuan et al., 2004), they were not included because of their complex mechanism of action. Indeed, the cytotoxicity of Dp44mT involves not only iron chelation but also the generation of cytotoxic radicals from its metal complex (Yuan et al., 2004). This would preclude interpretation of the effects caused by iron depletion alone.

Whole-Genome Gene Array Reveals Novel Genes Up- and Down-Regulated by Iron Depletion

Gene array analysis after a 24-h incubation with DFO (250 μM) or 311 (25 μM) demonstrated that there were 15 common genes significantly ($p < 0.05$) up-regulated and one common gene significantly ($p < 0.05$) down-regulated (Table 2) by both chelators. The expression of these genes was confirmed to be significantly altered by RT-PCR and the extent of the alteration is listed in Table 2. Because the array can be considered to be only a screening technique, its analysis alone was not considered definitive proof of differential expression. Rather, as stated under *Materials and Methods*, evidence of significant differential expression was obtained from RT-PCR assessment of samples used for the microarray and also at least three independent samples.

Of the 15 up-regulated genes, only two were previously reported to be regulated in response to iron. These include the *growth differentiation factor 15* (*GDF15*) gene, which plays a role in regulating the hormone of iron metabolism, hepcidin (Lakhal et al., 2009), and the tumor growth and metastasis suppressor gene *NDRG-1* (Le and Richardson, 2004). The only gene commonly down-regulated by both DFO or 311 was *amyloid β (A4) precursor protein* (*APP*) gene that contains a type II IRE-motif in its 5′-UTR (Rogers et al., 2002). The down-regulation of APP mRNA was also confirmed by RT-PCR, although the magnitude of the decrease was not as marked as that found in the array (Table 2).

In general, after 24-h incubation with DFO or 311, the extent of up- or down-regulation was similar in these chelators (Table 2). This is explained by the fact that although there was an initial kinetic block to the entry of DFO into cells, it does gain access after longer incubations of 24 h to chelate iron (Richardson et al., 1994). Moreover, because

TABLE 1
Sequences and accession number of primers used to amplify human and mouse transcripts via RT-PCR.

Organism & Pair No.	Primer Name	Accession No.	Oligonucleotides (5′-3′)		Product Size
			Forward	Reverse	
					bp
Homo sapiens					
1	*APP*	NM_000484	GCGCAGAACAGAAGGACA	GGCATCAACAGGCTCAAC	414
2	*BHLBH2*	NM_003670	GGATCTCCTACCCGAACA	TCTCCCATCGTGAACCTG	519
3	*BIM*	NM_138621.2	CCTACCTCCCTACAGACA	AGTAAGCGTTAAACTCGTC	391
4	*BNIP3*	NM_004052	ACAACCTCCACCAGCACC	AGCAAGAAGAGTTTAGTGTACGAAC	623
5	*BNIP3L*	NM_004331	GTATCTGTAGCCCTAACAC	ATAACGATACAAAGTGCTAACTAAA	438
6	*CCNG1*	NM_004060.3	ACACGATAATGGCCTCAG	ACATGCCTTCAGTTGAGC	448
7	*CCNG2*	NM_004354.1	ACCATCTGTATTAGCCTTGT	GTGTTTGTGCCACTTTGA	413
8	*CITED2*	NM_006079.3	GGCGGCTCTGGCAGCAGCTC	CGGGCAGCTCCTTCGTGCGG	205
9	*E2IG5*	NM_014367	AAAGGAAGATGAAATCCCAGAG	TTTCTCCAAAAGGCTGAGAGGCTAC	459
10	*EGR1*	NM_001964.2	GACCTGAAGGCCCTCAATAC	CATCGCTCCTGGCAAACT	322
11	*ERO1L*	NM_014584	TGGCTTCTGGTCAAGGGAC	CCAAATGCGTTGAATAATGATACTA	682
12	*ERRFI1*	NM_018948	GTTGCGTCTTGTTGAGGG	GTGAGAGTTTTCAAAGCAGAAAGAT	487
13	*GDF15*	NM_004864	AGAAGTGCGGCTGGGATC	CAAGTCATCATAGGTCTGGAGC	628
14	*HIG2*	NM_013332	TGAGTTTTGTGGCGGGAAGC	ATGGAAGGAGGTCTTATCACATGCT	389
15	*JMJD1A*	NM_018433.3	ACAACGGAGAAGATAAGGG	CCAGAGCTGATACGAGGT	696
16	*Loc401152*	NM_001001701	CAGGAGCGAGATGGAGGTG	TAAGAACTTAGCTAGGAATGGGTGA	416
17	*NDRG-1*	NM_006096	CCCTCGCGTTAGGCAGGTGA	AGGGGTACATGTACCCTGCG	370
18	*ODC1*	NM_002539	ATTGGCGGTGGCTTTCCT	GCAACAGTGTAAGCGCCCATGTTTT	476
19	*p53*	NM_000546	ACCCAGGTCCAGATGAAG	CACTCGGATAAGATGCTGA	422
20	*PNRC1*	NM_006813.1	GAGATGGCCCGTGTCTGA	AAGGTGGATCACTAAACTTTGC	678
21	*PPM1D*	NM_003620	CACAACCTCACAGCGAAAG	ACACAGGTCACTATGAAAATACATC	586
22	*TFRC*	NM_003234	GCTCGGCAAGTAGATGGC	TTGATGGTGCTGGTGAAG	359
23	*β-actin*	X00351	CCCGCCGCCAGCTCACCATGG	AAGGTCTCAAACATGATCTGGGTC	397
Mus musculus					
24	*Bnip3*	BC085237	AACAACAACTGCGAGGAA	TAGGGTGTCTGAAATGGAAC	439
25	*Ero1l*	AF144695	CCAGACGCTTGGAGGATA	GGCATATTTGCGATCAGT	686
26	*Gdf15*	BC067248	TGCTACTCCGCGTCAACC	GCTCCAGCCCAAGTCTTCA	359
27	*Hif-1α*	NM_010431	CGAGAAGAAAAAGATGAGTTCTGAACGTCG	CTGGATGCCGGTGGTCTAGACAG	210
28	*Ndrg-1*	NM_010884	TGCTTGCTCATTAGGTGTGTGATAGC	CCATCCTGAGATCTTAGAGGCAGC	581
29	*Ppm1d*	NM_016910	GAGTGAACCGAGTAGTTTGG	TGACTTGATTGGTGGTGTAG	545
30	*Vegf-1*	M95200.1	CCATGCCAAGTGGTCCCAG	GTCTTTCTTTGGTCTGCATTCACAT	346
31	*Tfrc*	NM_011638	TCCCGAGGGTTATGTGGC	GGCGGAAACTGAGTATGATTGA	324
32	*β-actin*	BC040513	CTTCCTTCTTGGGTATGGAATCCTGT	CTCAGGAGGAGCAATGATCTTGATCTTC	212

bp, base pair(s).

DFO was used at a higher concentration than 311 (250 versus 25 μM) to ensure intracellular chelation (see *Materials and Methods*), this also probably led to a response of a similar magnitude.

Apart from the 16 genes that were significantly up- or down-regulated by both DFO and 311 (Table 2), there were another 20 and 30 genes that were significantly down- or up-regulated by DFO or 311 alone, respectively (Tables 3 and 4). Other commonly known iron-regulated genes such as *TfR1* were not listed in these tables because the fold change was not sufficiently significant compared with the control. This provides an indication of the very high stringency used to process these array data. However, *TfR1* was identified by the array to be up-regulated by iron depletion (the average fold change for 311 and DFO from three different array probes for each chelator were $\log_2$ 1.81 and 1.95, respectively; http://www.ncbi.nlm.nih.gov/geo/). As a positive control, in all samples, RT-PCR demonstrated that TfR1 mRNA was shown to be significantly up-regulated by 311 (25 μM) and DFO (250 μM) after a 24-h incubation at 37°C.

The complete list of genes (Tables 2–4) in which expression was significantly altered after incubation with chelators was then assessed using gene ontology software. This was done to determine whether iron depletion affected any specific biological processes in particular (Table 5). The classification of these processes was defined by the functional annotation of genes assigned by the Gene Ontology Consortium (Suryo Rahmanto et al., 2007). Examining the processes associated with the differentially up-regulated genes induced by 311 and DFO demonstrated that, in general, each chelator had a similar effect (Table 5). For both chelators, the largest proportion (28–30%) of up-regulated genes was of "unknown

TABLE 2
Common statistically significant ($p < 0.05$) alterations in the up- and down-regulation of genes after incubating MCF-7 cells for 24 h at 37°C with 311 (25 μM) or DFO (250 μM).

Affymetrix ID	Gene Title	Gene Symbol	Gene Array ($\log_2$)		RT-PCR ($\log_2$)	
			311	DFO	311	DFO
Up-regulated genes						
201170_s_at	Basic helix-loop-helix domain containing, class B, 2	*BHLHB2*	4.01	3.82	2.89	2.08
201848_s_at	BCL2/adenovirus E1B 19-kDa interacting protein 3	*BNIP3*	3.38	3.33	3.31	3.82
221479_s_at	BCL2/adenovirus E1B 19-kDa interacting protein 3-like	*BNIP3L*	3.48	3.55	3.48	2.87
207980_s_at	Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain, 2	*CITED2*	3.63	3.65	4.13	2.90
220942_x_at	Growth and transformation-dependent protein	*E2IG5*	2.43	2.35	1.18	1.75
227404_s_at	Early growth response 1	*EGR1*	4.07	3.88	1.08	1.21
222646_s_at	ERO1-like (*S. cerevisiae*)	*ERO1L*	2.29	2.22	3.72	6.14
224657_at	ERBB receptor feedback inhibitor 1	*ERRFI1*	2.93	3.25	0.79	0.49
221577_x_at	Growth differentiation factor 15	*GDF15*	2.78	2.87	1.25	2.38
218507_at	Hypoxia-inducible protein 2	*HIG2*	3.42	3.21	0.43	0.64
212689_s_at	Jumonji domain containing 1A	*JMJD1A*	3.31	3.40	0.99	0.23
224602_at	HCV F-transactivated protein 1	*LOC401152*	1.99	2.17	2.73	1.46
200632_s_at	N-myc downstream regulated gene 1	*NDRG-1*	3.73	3.90	2.00	3.17
209034_at	Proline-rich nuclear receptor coactivator 1	*PNRC1*	3.37	3.41	1.73	1.53
204566_at	Protein phosphatase 1D magnesium-dependent, δ isoform	*PPM1D*	1.50	1.61	0.44	0.36
Down-regulated genes						
222013_x_at	Amyloid β (A4) precursor protein (peptidase nexin-II, Alzheimer disease)	APP	−3.72	−4.02	−0.98	−0.72

TABLE 3
Statistically significant ($p < 0.05$) down-regulated genes after incubating MCF-7 cells with 311 (25 μM) or DFO (250 μM) for 24 h at 37°C.

Affymetrix ID	Gene Title	Gene Symbol	Ratio ($\log_2$)	
			311	DFO
243958_at	Mannosyl (α-1,3-)-glycoprotein β-1,4-N-acetylglucosaminyltransferase, isozyme A	*MGAT4A*	−6.16	
242396_at	LOC440312	*LOC440312*	−4.16	
207090_x_at	Zinc finger protein KIAA0961	*KIAA0961*	−4.04	
228762_at	Lunatic fringe homolog (*Drosophila*)	*LFNG*	−3.99	
242828_at	Fidgetin	*FIGN*	−3.61	
204554_at	Protein phosphatase 1, regulatory subunit 3D	*PPP1R3D*	−3.25	
232075_at	WD repeat domain 61	*WDR61*	−3.14	
222760_at	Zinc finger protein 703	*ZNF703*	−3.01	
200768_s_at	Methionine adenosyltransferase II, alpha	*MAT2A*	−2.77	
46665_at	Sema domain, immunoglobulin domain (Ig), transmembrane domain (TM) and short cytoplasmic domain (semaphorin) 4C	*SEMA4C*	−2.62	
201368_at	Zinc finger protein 36, C3H type-like 2	*ZFP36L2*	−2.57	
222504_s_at	COX4 neighbor	*COX4NB*		−5.17
221909_at	Hypothetical protein FLJ14627	*FLJ14627*		−4.72
218300_at	Chromosome 16 open reading frame 53	*C16orf53*		−4.37
219451_at	Methionine sulfoxide reductase B2	*MSRB2*		−4.27
225834_at	Family with sequence similarity 72, member A	*FAM72A*		−4.00
202240_at	Polo-like kinase 1 (*Drosophila*)	*PLK1*		−3.57
202870_s_at	CDC20 cell division cycle 20 homolog (S. cerevisiae)	*CDC20*		−3.48
200790_at	Ornithine decarboxylase 1	*ODC1*		−3.38
207165_at	Hyaluronan-mediated motility receptor (RHAMM)	*HMMR*		−3.33

function." The second largest group of up-regulated genes (16–17%) after incubation with DFO or 311 were those affecting "nucleic acid-binding," whereas the third largest group of up-regulated genes (11%) belonged to the group of "nonreceptor Ser/Thr protein kinases" (Table 5).

Some differences between chelators were also observed, with 6% of up-regulated genes in the 311-treated cells belonging to the group of "guanyl nucleotide exchange factor" genes, 4% being "protein kinase" genes, 3% being "select regulatory molecules," and 3% being "signaling molecule genes." None of these gene classes was identified as up-regulated in DFO-treated cells. Likewise, "microtubule-binding motor binding" genes (4%), "DNA helicase" genes (3%), "exoribonuclease genes" (3%) and "receptor genes" (3%) were identified in DFO-treated cells, whereas none of these were identified to be up-regulated in cells incubated with 311 (Table 5).

The major group of genes down-regulated by both DFO and 311 (20 and 24%, respectively) was the "nucleic acid-binding" class (Table 5). The second group of genes commonly down-regulated by DFO and 311 were the "KRAB box transcription factor" series, which represented 12 and 16% of all genes, respectively. As found for the up-regulated genes, there was some discordance in gene expression between the two chelators. For instance, only in 311-treated cells was there a decrease in the expression of "G-protein modulator" genes, which represented 12% of the total (Table 5). Given the large number of genes identified as being regulated by iron chelation, we concentrated on those commonly and significantly modified by both DFO and 311 (Table 2).

Differential Gene Expression Depends on Chelator Concentration, and Incubation Time and Is Reversible by Adding Iron

From the 16 genes showing significantly altered expression after incubation with either DFO or 311 and then independently confirmed by RT-PCR (Table 2), we chose seven to further investigate and characterize in detail. The seven genes assessed (i.e., *BNIP3*, *EGR1*, *ERO1L*, *GDF15*, *NDRG-1*, *PPM1D*, and *PNRC1*) were chosen on the basis of 1) their functional relevance to cellular proliferation, iron metabolism, and cellular survival and 2) whether antibodies were available for assessing protein levels.

A dose- and time-dependent response was observed for all seven genes, expression increasing as a function of chelator concentration (Fig. 1B) and incubation time (Fig. 1C). For both *EGR1* and *NDRG-1*, a slight decrease in expression relative to the control was repeatedly observed at 311 concentrations between 0.25 and 1.0 μM (Fig. 1B). Collectively, these data confirmed the results of the gene array that was done at one concentration (311, 25 μM; DFO, 250 μM) and time point (24 h; Table 2).

It was also important to assess whether the alteration in gene expression after iron depletion using DFO or 311 could be reversed by the addition of iron as ferric ammonium citrate (FAC; 100 μg/ml), that donates iron to cells (Le and Richardson, 2004). This was necessary to demonstrate that the effect observed was due to iron depletion per se and could be reversed by adding iron. For all seven genes assessed, 311 or DFO significantly ($p < 0.05$) increased expression, and

TABLE 4
Statistically significant ($p < 0.05$) up-regulated genes after incubating MCF-7 cells for 24 h at 37°C with either 311 (25 μM) or DFO (250 μM).

Affymetrix ID	Gene Title	Gene Symbol	Ratio ($\log_2$)	
			311	DFO
202912_at	Adrenomedullin	*ADM*	5.96	
203725_at	Growth arrest and DNA-damage-inducible, α	*GADD45A*	4.23	
202464_s_at	6-Phosphofructo-2-kinase/fructose-2,6-biphosphatase 3	*PFKFB3*	4.16	
202887_s_at	DNA-damage-inducible transcript 4	*DDIT4*	3.37	
201464_x_at	v-Jun sarcoma virus 17 oncogene homolog (avian)	*JUN*	3.36	
223394_at	SERTA domain containing 1	*SERTAD1*	3.31	
211456_x_at	Similar to 60S ribosomal protein L35	*LOC440737*	3.17	
203394_s_at	Hairy and enhancer of split 1 (*Drosophila*)	*HES1*	3.14	
208581_x_at	Metallothionein 1X	*MT1X*	2.98	
203282_at	Glucan (1,4-α-), branching enzyme 1 (glycogen branching enzyme, Andersen disease, glycogen storage disease type IV)	*GBE1*	2.93	
201846_s_at	RING1 and YY1 binding protein	*RYBP*	2.66	
202769_at	Cyclin G2	*CCNG2*	2.51	
212185_x_at	Metallothionein 2A	*MT2A*	2.22	
201406_at	Ribosomal protein L36a	*RPL36A*	0.43	
200869_at	ribosomal protein L18a similar to ribosomal protein L18a;60S ribosomal protein L18a	*RPL18ALOC390354*	0.34	
201010_s_at	Thioredoxin interacting protein	*TXNIP*		3.06
217996_at	Pleckstrin homology-like domain, family A, member 1	*PHLDA1*		2.90
227897_at	RAP2B, member of RAS oncogene family	*RAP2B*		2.65
200738_s_at	Phosphoglycerate kinase 1	*PGK1*		1.38
208980_s_at	Ubiquitin C	*UBC*		1.15
200650_s_at	Lactate dehydrogenase A	*LDHA*		0.88
217733_s_at	Thymosin, β10	*TMSB10*		0.83
200966_x_at	Aldolase A, fructose-bisphosphate	*ALDOA*		0.63
200748_s_at	Ferritin, heavy polypeptide 1	*FTH1*		0.63
203012_x_at	Similar to 60S ribosomal protein L23a	*LOC130773*		0.53
206559_x_at	Eukaryotic translation elongation factor 1 α1	*EEF1A1*		0.49
205009_at	Trefoil factor 1 (breast cancer, estrogen-inducible sequence expressed in)	*TFF1*		0.48
212391_x_at	Ribosomal protein S3A	*RPS3A*		0.47
200834_s_at	Ribosomal protein S21	*RPS21*		0.47
200088_x_at	Ribosomal protein L12	*RPL12*		0.47

this was reversed to control levels or in some cases to significantly less ($p < 0.05$) than the control by a secondary incubation with FAC (Fig. 2). Secondary incubation with CON alone also reduced the chelator-mediated increase in expression, because the medium also contains some iron.

The reversibility of gene expression mediated by adding iron demonstrated that the up-regulation was due to iron depletion and not to some other property of the chelator. However, the extent of regulation was more notable for some genes (e.g., *BNIP3*) than others (e.g., *ERO1L*). The pattern of regulation observed after iron depletion followed by iron supplementation was similar to that of the positive controls, *TfR1* and *NDRG-1*, which are known iron-regulated genes (Le and Richardson, 2004) (Fig. 2).

Regulation of Gene Expression by the IRE-IRP Mechanism, HIF-1α, and p53

Regulation via the IRE-IRP Mechanism. A number of mechanisms could induce alterations in gene expression after iron depletion, including the IRP-IRE interaction (Sanchez et al., 2007). In addition, transcriptional processes mediated by HIF-1α and/or p53 have been shown to be regulated by iron (An et al., 1998; Liang and Richardson, 2003) and could also be involved. In our studies, three online software packages [m-fold (http://mfold.bioinfo.rpi.edu/cgi-bin/rna-form1.cgi), RNAfold (http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi), and the University of Wuerzburg Bioinformatics RNA Analyser (http://www.biozentrum.uni-wuerzburg.de/rnaanalyzer.html)] were used to assess the presence of IREs in the 3′-UTRs of the 15 genes identified to be significantly up-regulated after iron depletion by both chelators (Table 2).

Stem-loop structures were identified in the 3′-UTR of *NDRG-1* and *HIG2* (Fig. 3A). However, none of these was similar to those previously characterized within the UTRs of *TfR1*, *ferritin etc.* (Sanchez et al., 2007). Furthermore, none of the stem-loops identified contained the conserved, canonical IRE containing the six-nucleotide loop 5′-CAGWGH-3′, where W is adenosine or uridine and H is adenosine, cytosine, or uridine (Sanchez et al., 2007). Moreover, although the stem was thermodynamically stable in the *NDRG-1* and *HIG2* structures (Fig. 3A), it was not typical of that in other IREs (e.g., see *TfR1*; Fig. 3A). In fact, in the canonical IRE, the apical loop is found on a stem of five paired nucleotides containing a small asymmetrical bulge with an unpaired cytosine on the 5′-strand and an additional lower stem of variable length (Sanchez et al., 2007). Hence, IREs are highly conserved structures, and the stem-loops found in *NDRG-1* and *HIG2* would not be conducive to IRP-binding (Sanchez et al., 2007).

Regulation via HIF-1α. All 16 genes regulated by both chelators (Table 2) had potential hypoxia response elements (G/C/T ACGTGC G/C) (Liu et al., 1995) within their promoters as indicated by analysis using the Genomatix program (Suite 3; Genomatix, Munich, Germany) (Table 6). Furthermore, evidence from the literature suggested regulation of these genes by HIF-1α under a variety of conditions (Table 6). Considering this, the role of HIF-1α in the regulation of the seven genes up-regulated by iron chelation (Fig. 1, B and C) was examined using HIF-1α knockout [*HIF-1α*(−/−)] MEFs compared with their wild-type controls [*HIF-1α*(+/+); Fig. 3B]. As a control, assessment of *HIF-1α* mRNA was examined, which demonstrated that it was only present in *HIF-1α*(+/+) cells.

Incubation of MEFs with DFO (250 μM) or 311 (25 μM) led to an overall expression response to the iron chelators (Fig. 3B) similar to that found in MCF7 breast cancer cells (Fig. 2B). In fact, these chelators significantly ($p < 0.01$) increased *BNIP3*, *EGR1*, and *ERO1L* expression in *HIF-1α*(+/+) cells relative to cells treated control medium alone (CON), whereas no significant increase was found in *HIF-1α*(−/−) cells incubated under the same conditions (Fig. 3B). This indicated that an HIF-1α-dependent mechanism was involved in their up-regulation after iron chelation. In terms of the effect of hypoxia (0.5% O_2), *BNIP3* and *ERO1L* expression were significantly ($p < 0.01$) increased in *HIF-1α*(+/+) cells only. In contrast, hypoxia significantly ($p < 0.05$) increased *EGR1* in both *HIF-1α*(+/+) and *HIF-1α*(−/−) cells.

For *NDRG-1* and *PNRC1*, significant up-regulation occurred after incubation with chelators or hypoxia in the presence or absence of HIF-1α (Fig. 3B). This demonstrated that HIF-1α-dependent and -independent mechanisms could be involved in regulating these genes. Chelators significantly ($p < 0.05$) up-regulated *GDF-15* and *PPM1D* expression in the presence or absence of HIF-1α, whereas hypoxia had no effect in the case of *PPM1D* but significantly ($p < 0.05$) increased *GDF15* in *HIF-1α*(+/+) cells only.

Regulation via p53. Assessment of p53 response elements (A/C A/C A/C C A/T A/T G G/T G/T G/T) (el-Deiry et al., 1992) in the promoter of the 16 genes that were regulated by both chelators (Table 2) using the Genomatix program led to their identification in the promoters of *APP*, *EGR1*, *GDF15*,

TABLE 5

Biological processes associated with statistically significant ($p < 0.05$) differentially expressed genes identified from the Affymetrix Human Genome U133 Plus 2.0 gene array after incubation of MCF-7 cells for 24 h at 37°C with 311 (25 μM) or DFO (250 μM).

	311	DFO
	%	
Up-regulated genes		
Unknown functions	28	30
Nucleic acid-binding	16	17
Nonreceptor serine/threonine protein kinase	11	11
Transcription factor	8	7
Other functions	7	7
Ribosomal protein	5	7
Small GTPase	5	5
Zinc finger transcription factor	4	3
Guanyl-nucleotide exchange factor	6	
Protein kinase	4	
Select regulatory molecule	3	
Signaling molecule	3	
Microtubule-binding motor protein		4
DNA helicase		3
Exoribonuclease		3
Receptor		3
Down-regulated genes		
Nucleic acid-binding	24	20
KRAB box transcription factor	16	12
Zinc finger transcription factor	11	12
Other functions	11	7
Guanyl-nucleotide exchange factor	10	8
Transcription factor	10	8
Unknown functions	6	9
G-protein modulator	12	
Non–motor actin-binding protein		8
Nonreceptor serine/threonine protein kinase		9
Transferase		7

NDRG-1, and *PPM1D* (Table 6). This was supported by a literature search, which indicated that these genes can be regulated by p53 via a variety of stimuli (Table 6). Because p53 expression can be regulated by intracellular iron levels (An et al., 1998; Liang and Richardson, 2003), we examined the role of p53 in the up-regulation of the seven genes examined after iron depletion (Fig. 1, B and C). This was done by implementing H1299 cells (*p53*-null) transfected with wild-type p53 under the control of a Tet-responsive promoter.

Only cells containing the *p53* construct incubated with Tet led to detectable p53 mRNA (Fig. 4). The addition of Tet to H1299 transfected with the empty vector alone led to no p53 expression. It is noteworthy that incubation of H1299 cells transfected with wild-type p53 together with the chelators increased *p53* expression. The mechanism involved in this effect is unclear but may involve the effect of iron depletion on stabilizing p53 mRNA leading to increased translation. The expression of *GDF15*, *NDRG-1*, and *PPM1D* was significantly ($p < 0.05$) increased in the presence of chelators in cells expressing p53, although no influence was observed in cells not expressing this transcription factor (Fig. 4). This suggested that for these genes the increase in expression was p53-dependent. Surprisingly, in *p53*-null H1299 cells, there was no up-regulation of *NDRG-1* after incubation with chelators (Fig. 4). This was unexpected, because these cells are HIF-1α(+/+) and should respond as in the studies shown in

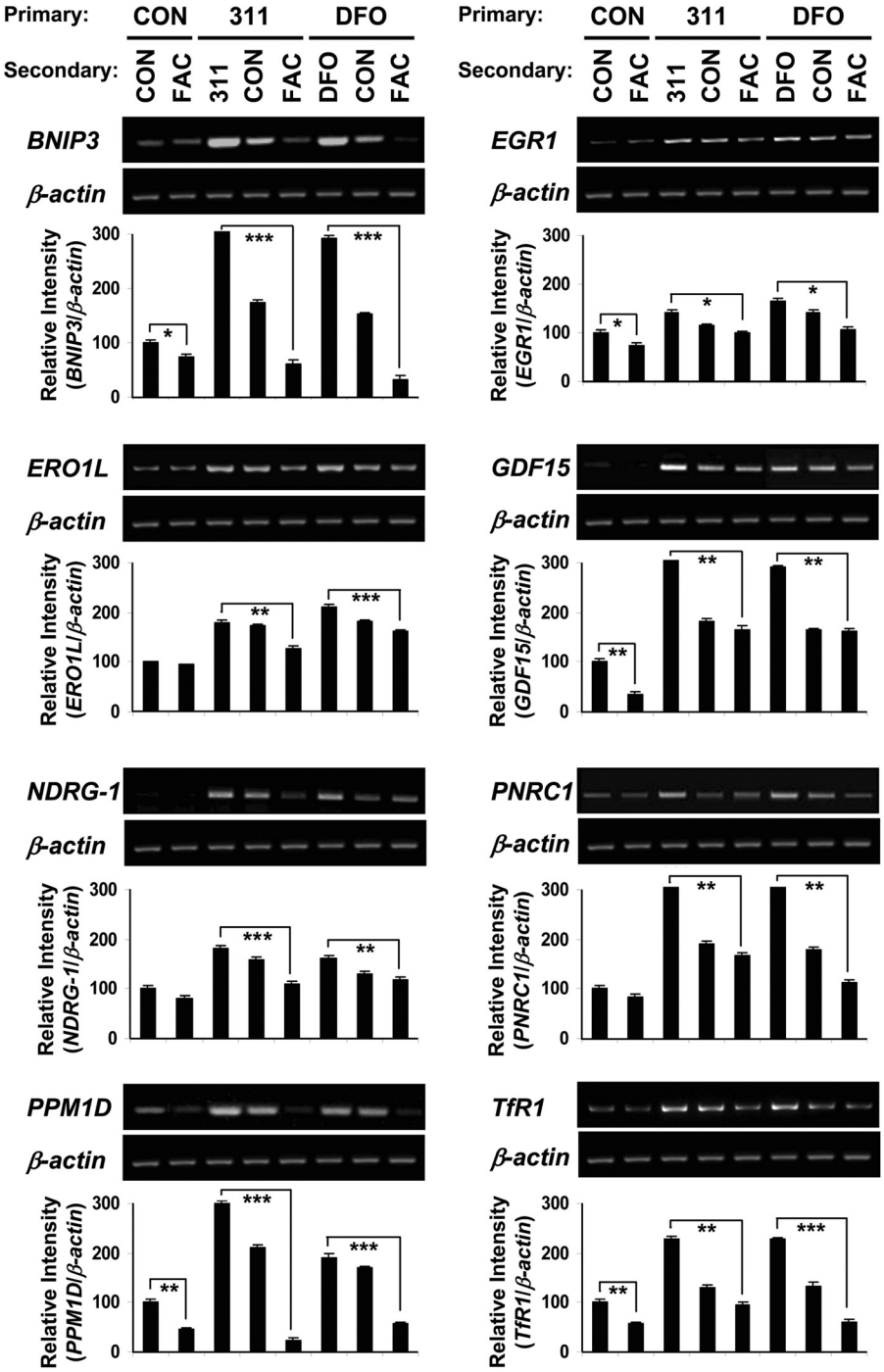

Fig. 2. Chelator-mediated increase in gene expression is reversible by reincubation with the soluble iron source FAC. MCF-7 cells were incubated for 18 h at 37°C with CON alone or CON containing DFO (250 μM) or 311 (25 μM) (primary incubation). This medium was then removed, and the cells were reincubated for 22 h at 37°C with either CON alone or CON containing either FAC (100 μg/ml), DFO (250 μM), or 311 (25 μM) (secondary incubation). The mRNA expression of the seven genes examined in Fig. 1 was then examined in comparison with the known iron-regulated genes *NDRG-1* and *TfR1* (positive controls). Densitometry was performed and gene expression was then calculated relative to the *β-actin* control. Results are mean ± S.D. of three experiments. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

Fig. 3. However, this result may suggest that both p53 and HIF-1α are necessary for chelator-mediated up-regulation of *NDRG-1* in these cells. In fact, HIF-1α is known to be able to stabilize p53 (An et al., 1998), which may explain these

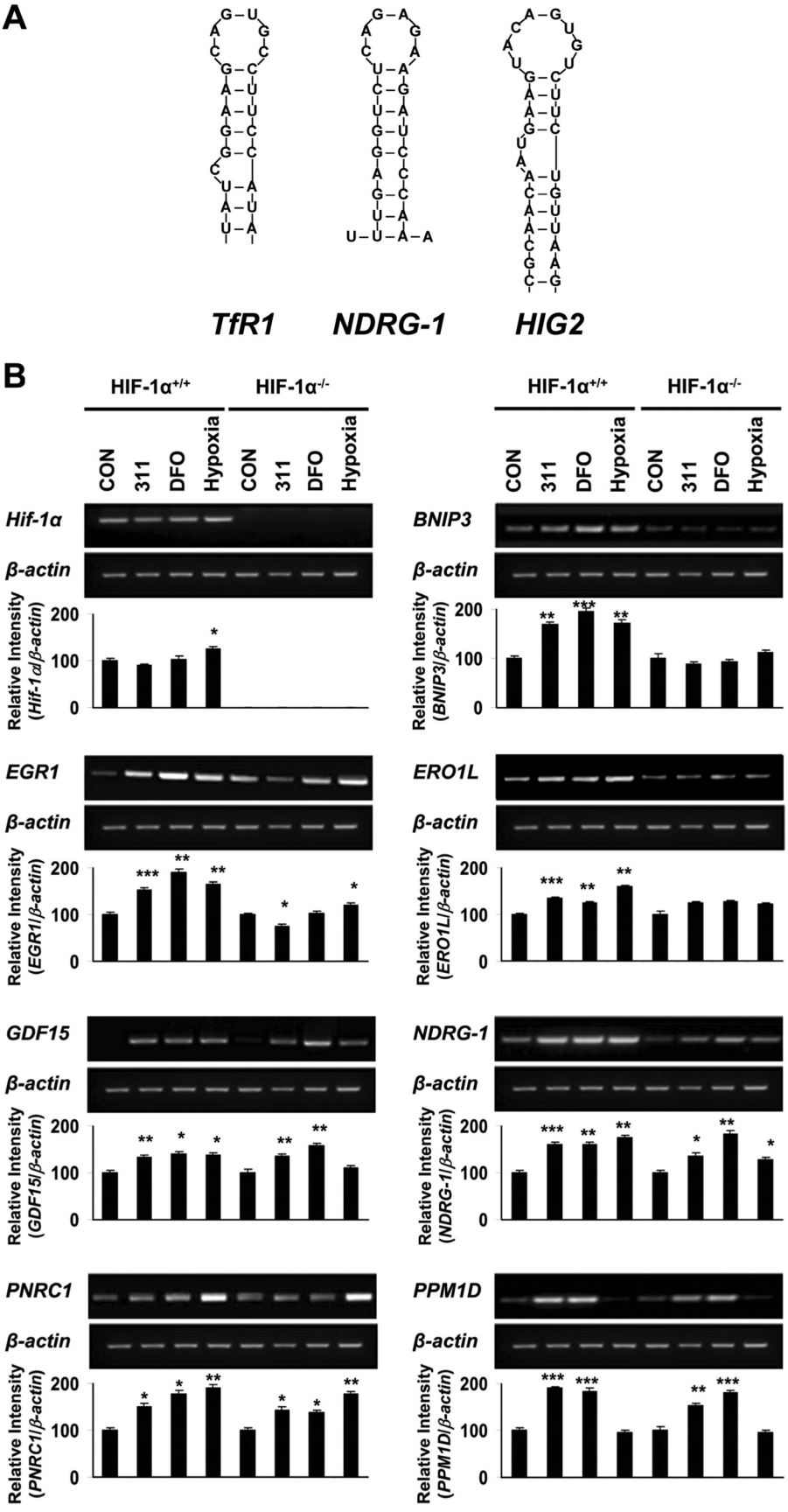

Fig. 3. A, diagram of one of the IREs in the 3′-UTR of *TfR1* mRNA in comparison with the stem loops in the 3′-UTR of *NDRG-1* and *HIG2*. B, examination of the role of HIF-1α in the chelator-mediated increase in gene expression. Wild-type [*HIF-1*α(+/+)] and *HIF-1*α-knockout [*HIF-1*α(−/−)] MEFs were incubated for 24 h at 37°C with either CON, DFO (250 μM), 311 (25 μM), or hypoxia (0.5% O_2), and their mRNA was extracted for analysis using RT-PCR (described under *Materials and Methods*). Densitometry was performed and gene expression then calculated relative to the *β-actin* loading control. Results are mean ± S.D. of three or four experiments. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ compared with the relevant CON alone for each cell type.

observations. For all other genes, their expression was increased after incubation with chelators in the presence or absence of p53, indicating p53-dependent and -independent mechanisms.

Iron-Regulated Gene Expression in Neoplastic Cells Compared with Normal Cells

Considering the significant ($p < 0.05$) differential regulation of 16 genes in Table 2 by both DFO and 311 in MCF-7 cells, studies assessed whether this effect was also observed in normal cells incubated with the same concentration of chelators (Fig. 5, A and B). In these experiments, we compared neoplastic MCF-7 cells with normal MRC-5 fibroblasts. This was done because we showed that proliferation of MCF-7 cells is highly sensitive to chelators relative to normal MRC-5 cells (Yuan et al., 2004) and that these ligands lead to iron mobilization from both cell types (Liang and Richardson, 2003; Yuan et al., 2004). Thus, an analysis of the expression of these 16 genes in both cell types may lead to improved understanding of the reason for the known antiproliferative efficacy of chelators against neoplastic relative to normal cells (Buss et al., 2003).

Five of the sixteen genes in Table 2 (namely *APP*, *CITED2*, *EGR1*, *GDF15*, and *PNRC1*) displayed differential regulation between MCF-7 and MRC-5 cells (Fig. 5A), whereas the remaining 11 genes showed similar up-regulation in both cell types (Fig. 5B). Hence, the differential gene expression observed between these cells could be important for understanding the sensitivity of cancer cells to chelators (Buss et al., 2003). Considering these results, it was essential to determine whether these differences in expression were evident in multiple tumor and normal cell types (Figs. 6 and 7).

To determine this, we examined the mRNA and protein expression of the five genes above that were differentially regulated between MCF-7 and MRC-5 cells in three other cancer cell lines (DMS53 lung cancer cells, SK-Mel-28 melanoma cells, SK-N-MC neuroepithelioma cells) and two other normal cell types (HUVECs and fibroblasts; Figs. 6 and 7).

At the mRNA level, *CITED2*, *EGR1*, *GDF15*, and *PNRC1* were significantly up-regulated by chelators to a significantly ($p < 0.05$) greater extent in neoplastic cells than in normal cells (Fig. 6). Differential regulation of these genes was even more marked when assessing protein levels, CITED2, EGR1, and GDF15 being significantly ($p < 0.005$) up-regulated by both chelators in neoplastic cells with either no effect or down-regulation occurring in normal cells (Fig. 7). The antibody available for PNRC1 did not lead to reproducible results; thus, these data were not included. For all cell types, the positive control *TfR1* was significantly up-regulated by chelators, demonstrating that iron depletion had occurred (Fig. 7).

It of interest that for the five genes analyzed in Figs. 6 and 7, mRNA levels were not always predictive of protein levels. For example, this is true of CITED2 and GDF15 in at least two of the three normal cell lines. Indeed, for the latter two molecules, protein levels were the opposite of what one would anticipate based upon RNA analyses. This difference can be rationalized by post-transcriptional and post-translational processing.

Discussion

Despite the fact that iron-deficiency anemia remains a severe global problem, little is known concerning the role of iron in regulating mammalian gene expression. Furthermore, the use of chelators for the treatment of iron overload disease and their use in the treatment of other conditions such as cancer (Buss et al., 2003), necessitates knowledge of how alterations in cellular iron levels affect gene expression. In fact, several chelators are being developed for cancer treatment (Buss et al., 2003); one of these, 3-aminopyridine-2-carboxaldehyde thiosemicarbazone (Triapine), has entered a variety of phase II clinical trials (Buss et al., 2003; Yu et al., 2009). Some of these investigations have shown that 3-aminopyridine-2-carboxaldehyde thiosemicarbazone is not highly effective and justifies the development of more effective ligands (e.g., Dp44mT) (Yu et al., 2009).

In this study, we used whole-genome gene array to assess alterations in gene expression after incubation with two well

TABLE 6

Genes regulated by p53 and HIF-1α

The presence (+) or absence (−) of p53 and/or HIF-1α, either in the literature (as regulating molecules) or in the sequence search using Genomatix [as p53 binding sequence or hypoxia response element (HRE)], is indicated.

Gene Symbol	Function	Literature			Genomatix	
		p53	HIF-1α	References	p53	HRE
APP	Cell proliferation and oxidative stress	+	+	Ozaki et al., 2006; Wang et al., 2006; Zhang et al., 2007.	+	+
BHLHB2	Cell growth and differentiation	−	+	Ivanova et al., 2001; Miyazaki et al., 2002; Chakrabarti et al., 2004; Currie et al., 2004; Yamada and Miyamoto, 2005	−	+
BNIP3	Apoptosis	−	+	Guo et al., 2001; Dayan et al., 2006; Lee and Paik, 2006	−	+
BNIP3L	Apoptosis	−	+	Fei et al., 2004	−	+
CITED2	Transcription and development	−	+	Freedman et al., 2003; van den Beucken et al., 2007.	−	+
E2IG5	Cell growth and differentiation	−	−		−	+
EGR1	Tumor suppressor	+	−	Yan et al., 1999; Liu et al., 2001; Rong et al., 2006	+	+
ERO1L	Disulfide bond formation	−	+	May et al., 2005.	−	+
ERRFI1	Tumor suppressor	−	+	Saarikoski et al., 2002; Packer et al., 2007	−	+
GDF15	Cell growth and differentiation	+	+	Morrish et al., 2001; Secchiero et al., 2006; Yang et al., 2006	+	+
HIG2	Autocrine growth factor	−	+	Denko et al., 2000; Togashi et al., 2005	−	+
JMJD1A	cell signaling	−	−		−	+
LOC401152	Cell cycle regulation, metabolism and cell apoptosis	−	−		−	+
NDRG-1	Metastasis suppressor	+	+	Le and Richardson, 2004; Stein et al., 2004	+	+
PNRC1	Tumor suppressor	−	−		−	+
PPM1D	Cell cycle regulation	+	−	Lu et al., 2004, 2005	+	+

characterized iron(III) chelators, DFO and 311, that have contrasting properties of cell permeability, iron chelation efficacy, and antiproliferative activity (Darnell and Richardson, 1999; Richardson and Bernhardt, 1999). For the first time, we identify a wide range of genes that have not been described previously as being regulated by intracellular iron levels. In fact, there were 16 genes significantly regulated by both 311 and DFO (Table 2) and a further 50 genes significantly regulated by one or the other chelator (Tables 3 and 4). These genes represent molecules with various functions, those of the nucleic acid-binding class being the most commonly up- or down-regulated (Table 5). Although DFO and 311 are well characterized iron(III) chelators, their different permeability characteristics and iron chelation efficacy (Darnell and Richardson, 1999; Richardson and Bernhardt, 1999) lead to some variation in response (Table 5).

Of the sixteen genes commonly and significantly regulated by both chelators (Table 2), seven were further characterized for reasons of practicality. All seven were confirmed to be reversibly regulated after iron depletion by the addition of FAC (Fig. 2), which is well known to replenish iron pools (Le and Richardson, 2004). Hence, the effects of the chelators on altering the expression of these genes cannot be interpreted to be due to another effect separate from iron binding.

By and large, the regulation of gene expression by iron in mammalian cells has been described to be via IRE-IRP and, to a lesser extent, HIF-1α mechanisms (Semenza, 1999; Sanchez et al., 2007). However, there is evidence that the transcription factor p53 is up-regulated by iron depletion (An et al., 1998; Liang and Richardson, 2003), and this could occur via HIF-1α, which stabilizes p53 (An et al., 1998). Furthermore, it has been shown that p53 can post-transcrip-

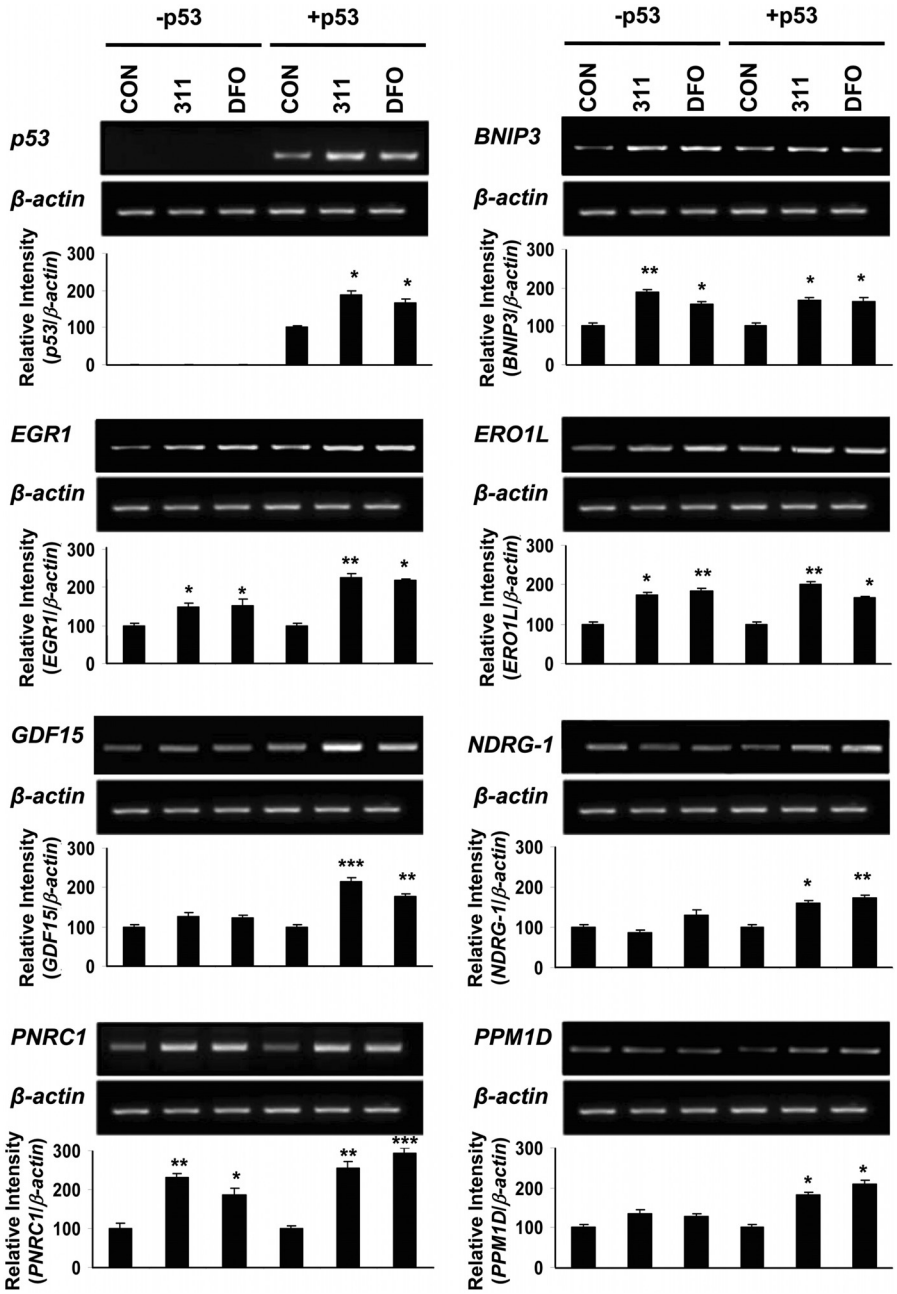

Fig. 4. Examination of the role of p53 in the chelator-mediated increase in gene expression. H1299 cells (*p53*-null) and H1299 cells stably transfected with a tetracycline-regulated p53 construct (Tet-on system) were incubated for 24 h at 37°C with either CON alone or CON containing DFO (250 μM) or 311 (25 μM). The mRNA was extracted, RT-PCR (described under *Materials and Methods*) and densitometry were performed, and gene expression was then calculated relative to the *β-actin* loading control. Results are mean ± S.D. of three or four experiments. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ compared with the relevant CON alone for each cell type.

tionally regulate iron homeostasis (Zhang et al., 2008). Hence, it was important to examine whether any of the alterations in gene expression observed could be due to these mechanisms. None of the up-regulated genes in Table 2 contained canonical IREs in the 3′ UTR; thus, IRE-IRP binding could probably not explain the up-regulation observed. However, both chelators led to significant up-regulation of three (*BNIP3*, *EGR1*, and *ERO1L*) of the seven genes characterized in Fig. 1, B and C, by a HIF-1α-dependent mechanism (Fig. 3B). It is noteworthy that these genes have been shown to be regulated by HIF-1α under other experimental conditions (Bruick, 2000; Gess et al., 2003; Tai et al., 2009).

The products of *BNIP3*, *EGR1*, and *ERO1L* are involved in apoptosis (Bruick, 2000), the increased expression of multiple tumor suppressors (TGFβ1, PTEN, p53, and fibronectin) (Baron et al., 2006) and redox homeostasis (Sevier and Kaiser, 2008), respectively. Thus, their increased expression could participate in the antiproliferative activity of iron chelators. Furthermore, EGR1 is necessary for *trans*-activation of the *HIF-1α* promoter (Sperandio et al., 2009), potentially resulting in a positive feed-forward loop in response to iron depletion, leading to increased HIF-1α, which then increases EGR1.

In additional studies, we examined the role of p53 in the response to iron depletion using H1299 cells (*p53*-null) transfected with wild-type p53. Chelator-mediated up-regulation by a p53-dependent mechanism was identified for *GDF15*, *NDRG-1*, and *PPM1D*. These genes were not up-regulated by the ligands in the absence of p53, indicating the specificity of the response. All of these genes contain a consensus p53-binding element within the promoter and have been shown previously to be regulated via p53 by a range of other stimuli (Table 6). Considering their functions, GDF15, NDRG-1, and PPM1D play roles in apoptosis (Jutooru et al., 2009), growth and metastasis suppression (Le and Richardson, 2004), and the regulation of p53, respectively (Lu et al., 2007). Hence, their effects on inhibiting tumor growth are notable when considering the antiproliferative activity of chelators.

Part of the rationale of this investigation was to decipher alterations in gene expression by chelators that could help explain the sensitivity of neoplastic cells to these agents relative to normal cells (Buss et al., 2003; Yu et al., 2009). In fact, *CITED2*, *EGR1*, *GDF15,* and *PNRC1*, were significantly up-regulated by DFO and 311 in four different neoplastic cell lines relative to three normal cell types. Each of these molecules has antiproliferative effects, and their collective activ-

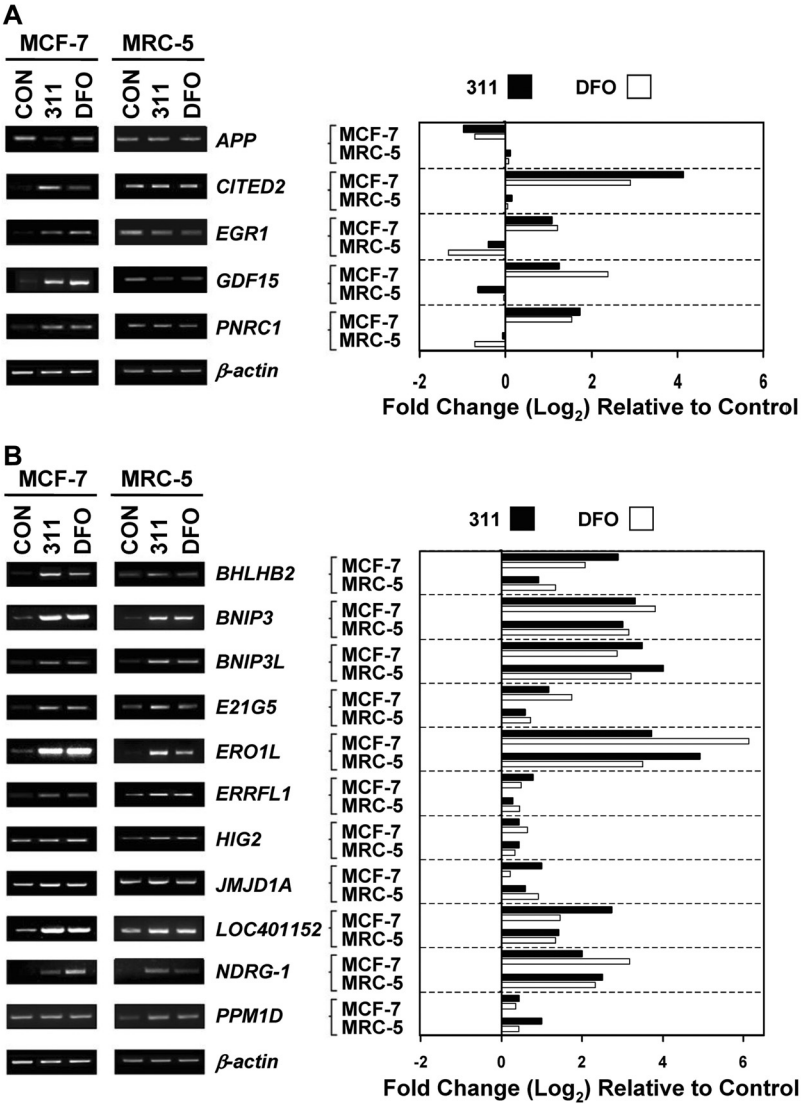

Fig. 5. Presence (A) or absence (B) of differential gene expression at the mRNA level between neoplastic (MCF-7 cells) and normal (MRC5 fibroblasts) after incubation for 24 h at 37°C with either CON alone or CON containing DFO (250 μM) or 311 (25 μM). The mRNA was extracted, RT-PCR and densitometry were performed, and gene expression ($\log_2$) was calculated relative to the *β-actin* loading control. Results are a typical experiment from two performed.

ity could be significant in terms of the antitumor efficacy of chelators (Buss et al., 2003). For instance, the transcriptional modulator CITED2 arrests cell growth and is also related to reduced expression of matrix metalloproteinase-13, which is linked to metastasis (Bai and Merchant, 2007). As described above, EGR1 and GDF15 have antitumor activity, and the chelator-mediated increase in expression is dependent on HIF-1α and p53, respectively. In fact, EGR1 is associated with susceptibility to apoptosis (Zagurovskaya et al., 2009) and expression of p21 (Choi et al., 2008), which inhibits proliferation. *GDF15* is also known as *nonsteroidal anti-inflammatory drug-activated gene-1* or macrophage inhibitory cytokine-1/prostate-derived factor and is a putative tumor suppressor (Yoshioka et al., 2008) the expression of which is increased by antitumor agents. As such, this molecule has been suggested to be a target for cancer treatment (Martinez et al., 2006). Furthermore, PNRC1 interacts with the Grb2-adaptor protein involved in growth factor/Ras-mediated pathways, and PNRC1 overexpression can inhibit cancer cell proliferation (Zhou et al., 2004).

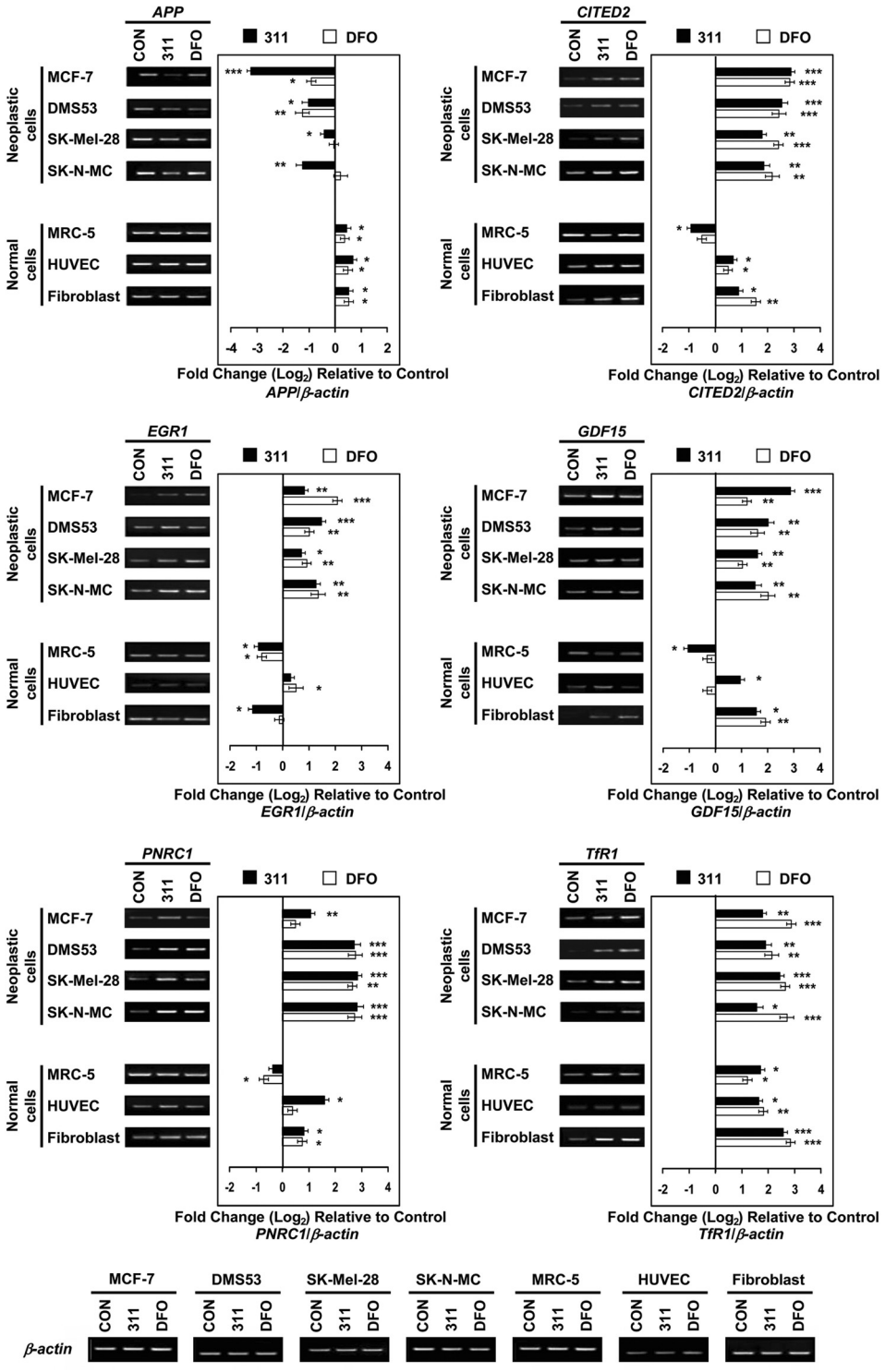

Fig. 6. Comparative data analysis of iron-regulated genes at the mRNA level showing differential expression between neoplastic cell lines (DMS-53, MCF-7, SK-Mel-28, and SK-N-MC) and normal cells (HUVECs, MRC-5, and fibroblasts) after incubation for 24 h at 37°C with either DFO (250 μM) or 311 (25 μM) compared with CON alone. The mRNA was extracted and RT-PCR and densitometry then performed. Gene expression ($\log_2$) was calculated relative to the *β-actin* loading control for each cell type and is placed at the bottom of the figure for clarity. The gel photographs are representative of at least three to six separate experiments performed, and the densitometry results are expressed as mean ± S.D. of three to six experiments. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ compared with the relevant CON for each cell type.

Apart from the up-regulation of the genes described above in neoplastic cells, it was also of interest that APP was generally expressed to a higher extent at the mRNA and protein levels in normal relative to tumor cells after incubation with chelators. Hence, the lower expression of APP after Fe chelation in tumor cells may lead to inhibition of proliferation, in that this molecule is known to promote cancer growth (Takayama et al., 2009).

A subset of the 16 genes identified in Table 2 were similarly regulated by iron chelation in both normal and neoplastic cell types (Fig. 5B). Considering this, after iron chelation therapy is given to patients for iron-overload disease, it can be expected that some of the alterations in gene expression reported herein could potentially be observed. Hence, the work described here is also important for understanding the response of both normal and neoplastic cells to iron chelation.

It is of interest that iron chelators affect a wide variety of targets, and the differential expression and function of the genes identified here must be considered in conjunction with the effects of the ligands on a variety of other molecules,

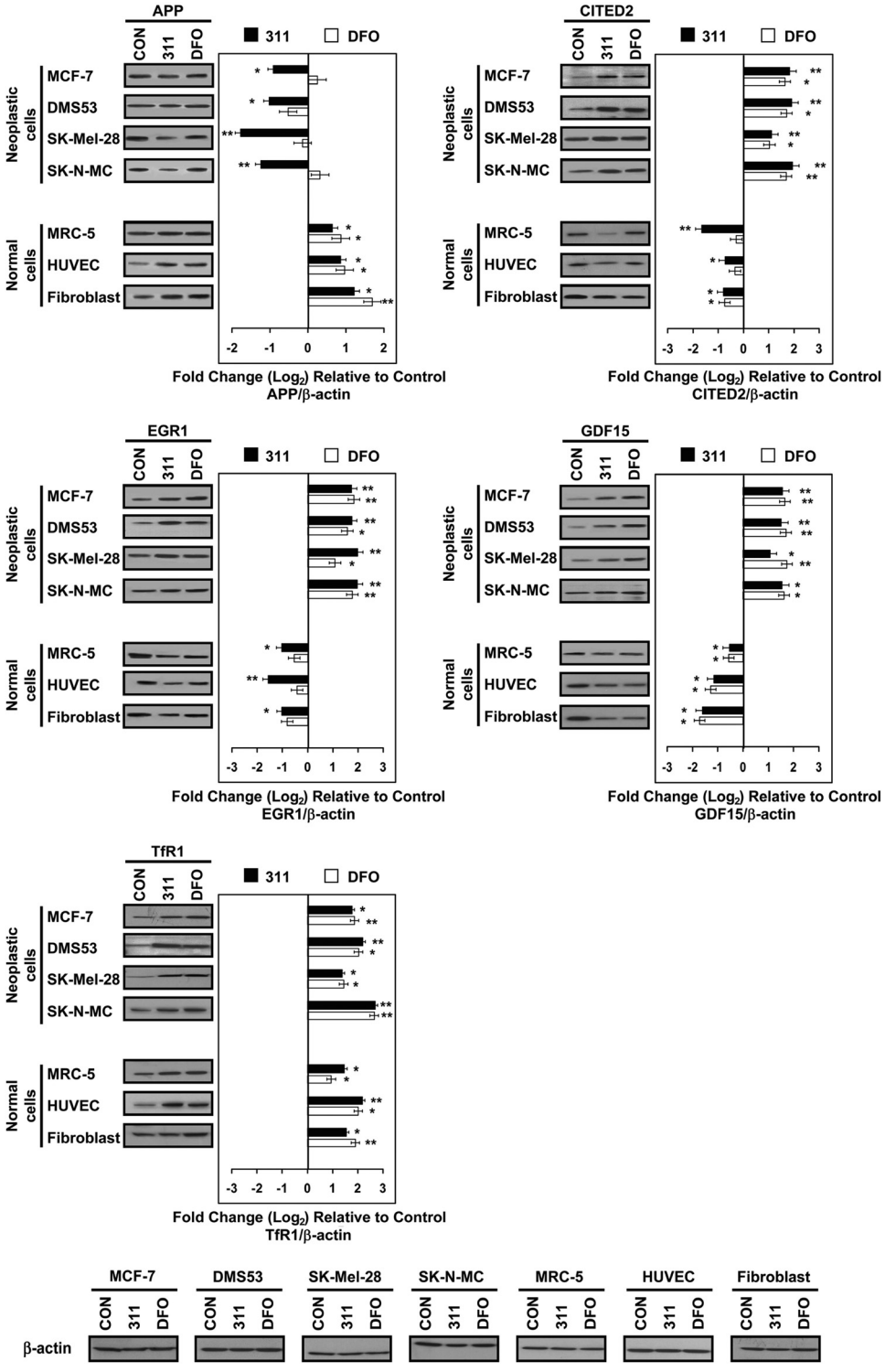

Fig. 7. Comparative data analysis of iron-regulated proteins showing differential expression between neoplastic cell lines (DMS-53, MCF-7, SK-Mel-28, and SK-N-MC) and normal cells (HUVECs, MRC-5, and fibroblasts) after incubation with either DFO (250 μM) or 311 (25 μM) for 24 h at 37°C compared with CON. The protein was extracted, and Western analysis and densitometry were then performed. Gene expression ($\log_2$) was calculated relative to the *β-actin* loading control for each cell type and is placed at the bottom of the figure for clarity. The photographs of Western blots are representative of at least three to six separate experiments performed, and the densitometry results are expressed as mean ± S.D. of three to six experiments). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ compared with the relevant CON for each cell type.

including cyclin D1, the cyclin-dependent kinase inhibitor $p21^{CIP1/WAF1}$, GADD45 (Darnell and Richardson, 1999; Gao and Richardson, 2001), ribonucleotide reductase, NDRG-1 (Le and Richardson, 2004), etc. The importance of iron chelators having multiple molecular targets could be significant for understanding why these agents inhibit tumor growth. In fact, the effect of iron depletion on activation of HIF-1α has been linked to the stimulation of tumor angiogenesis and resistance to chemotherapy. For example, Elstner et al., (2007) showed that DFO-induced iron depletion significantly enhanced glioblastoma cell invasion in vitro. However, apart from its tumor-stimulating activity, HIF-1α can also potentially have antitumor effects by up-regulating the pro-apoptotic molecule BNIP3 (Bruick, 2000) and the potent tumor suppressor p53 (An et al., 1998). The fact that potent iron chelators markedly prevent tumor growth in vivo (Buss et al., 2003; Yu et al., 2009) indicates that their antitumor effects rather than pro-tumor activity predominates. This may be true not only via the paradoxical activity of HIF-1α but also through the many other molecular targets of iron chelators (ribonucleotide reductase, etc.).

In conclusion, for the first time, we have identified a wide range of genes significantly regulated by chelation of cellular iron pools. In addition to iron-mediated regulation of expression of *BNIP3*, *EGR1*, and *ERO1L* via a HIF-1α-dependent mechanism, we also showed that p53 played a role in the iron-mediated regulation of *GDF15*, *NDRG-1*, and *PPM1D*. Sixteen genes were identified to be commonly regulated by DFO and 311. Of these, five were differentially regulated in tumor cells relative to normal cells. These molecules have antitumor functions, and their up-regulation could be significant in explaining the sensitivity of cancer cells to chelators. Moreover, these studies achieve a more comprehensive understanding of the diverse cellular responses to iron depletion.

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References

An WG, Kanekal M, Simon MC, Maltepe E, Blagosklonny MV, and Neckers LM (1998) Stabilization of wild-type p53 by hypoxia-inducible factor 1alpha. *Nature* **392:**405–408.

Bai L and Merchant JL (2007) A role for CITED2, a CBP/p300 interacting protein, in colon cancer cell invasion. *FEBS Lett* **581:**5904–5910.

Baron V, Adamson ED, Calogero A, Ragona G, and Mercola D (2006) The transcription factor Egr1 is a direct regulator of multiple tumor suppressors including TGFbeta1, PTEN, p53, and fibronectin. *Cancer Gene Ther* **13:**115–124.

Bianchi L, Tacchini L, and Cairo G (1999) HIF-1-mediated activation of transferrin receptor gene transcription by iron chelation. *Nucleic Acids Res* **27:**4223–4227.

Bruick RK (2000) Expression of the gene encoding the proapoptotic Nip3 protein is induced by hypoxia. *Proc Natl Acad Sci U S A* **97:**9082–9087.

Buss JL, Torti FM, and Torti SV (2003) The role of iron chelation in cancer therapy. *Curr Med Chem* **10:**1021–1034.

Chakrabarti J, Turley H, Campo L, Han C, Harris AL, Gatter KC, and Fox SB (2004) The transcription factor DEC1 (stra13, SHARP2) is associated with the hypoxic response and high tumour grade in human breast cancers. *Br J Cancer* **91:**954–958.

Chaston TB, Lovejoy DB, Watts RN, and Richardson DR (2003) Examination of the antiproliferative activity of iron chelators: multiple cellular targets and the different mechanism of action of triapine compared with desferrioxamine and the potent pyridoxal isonicotinoyl hydrazone analogue 311. *Clin Cancer Res* **9:**402–414.

Choi BH, Kim CG, Bae YS, Lim Y, Lee YH, and Shin SY (2008) p21 Waf1/Cip1 expression by curcumin in U-87MG human glioma cells: role of early growth response-1 expression. *Cancer Res* **68:**1369–1377.

Currie MJ, Hanrahan V, Gunningham SP, Morrin HR, Frampton C, Han C, Robinson BA, and Fox SB (2004) Expression of vascular endothelial growth factor D is associated with hypoxia inducible factor (HIF-1alpha) and the HIF-1alpha target gene DEC1, but not lymph node metastasis in primary human breast carcinomas. *J Clin Pathol* **57:**829–834.

Darnell G and Richardson DR (1999) The potential of iron chelators of the pyridoxal isonicotinoyl hydrazone class as effective antiproliferative agents III: the effect of the ligands on molecular targets involved in proliferation. *Blood* **94:**781–792.

Dayan F, Roux D, Brahimi-Horn MC, Pouyssegur J, and Mazure NM (2006) The oxygen sensor factor-inhibiting hypoxia-inducible factor-1 controls expression of distinct genes through the bifunctional transcriptional character of hypoxia-inducible factor-1alpha. *Cancer Res* **66:**3688–3698.

Denko N, Schindler C, Koong A, Laderoute K, Green C, and Giaccia A (2000) Epigenetic regulation of gene expression in cervical cancer cells by the tumor microenvironment. *Clin Cancer Res* **6:**480–487.

el-Deiry WS, Kern SE, Pietenpol JA, Kinzler KW, and Vogelstein B (1992) Definition of a consensus binding site for p53. *Nat Genet* **1:**45–49.

Elstner A, Holtkamp N, and von Deimling A (2007) Involvement of Hif-1 in desferrioxamine-induced invasion of glioblastoma cells. *Clin Exp Metastasis* **24:**57–66.

Fei P, Wang W, Kim SH, Wang S, Burns TF, Sax JK, Buzzai M, Dicker DT, McKenna WG, Bernhard EJ, and El-Deiry WS (2004) Bnip3L is induced by p53 under hypoxia, and its knockdown promotes tumor growth. *Cancer Cell* **6:**597–609.

Freedman SJ, Sun ZY, Kung AL, France DS, Wagner G, and Eck MJ (2003) Structural basis for negative regulation of hypoxia-inducible factor-1alpha by CITED2. *Nat Struct Biol* **10:**504–512.

Gao J and Richardson DR (2001) The potential of iron chelators of the pyridoxal isonicotinoyl hydrazone class as effective antiproliferative agents, IV: The mechanisms involved in inhibiting cell-cycle progression. *Blood* **98:**842–850.

Gess B, Hofbauer KH, Wenger RH, Lohaus C, Meyer HE, and Kurtz A (2003) The cellular oxygen tension regulates expression of the endoplasmic oxidoreductase ERO1-Lalpha. *Eur J Biochem* **270:**2228–2235.

Guo K, Searfoss G, Krolikowski D, Pagnoni M, Franks C, Clark K, Yu KT, Jaye M, and Ivashchenko Y (2001) Hypoxia induces the expression of the pro-apoptotic gene BNIP3. *Cell Death Differ* **8:**367–376.

Ivanova AV, Ivanov SV, Danilkovitch-Miagkova A, and Lerman MI (2001) Regulation of STRA13 by the von Hippel-Lindau tumor suppressor protein, hypoxia, and the UBC9/ubiquitin proteasome degradation pathway. *J Biol Chem* **276:**15306–15315.

Jutooru I, Chadalapaka G, Chintharlapalli S, Papineni S, and Safe S (2009) Induction of apoptosis and nonsteroidal anti-inflammatory drug-activated gene 1 in pancreatic cancer cells by a glycyrrhetinic acid derivative. *Mol Carcinog* **48:**692–702.

Lakhal S, Talbot NP, Crosby A, Stoepker C, Townsend AR, Robbins PA, Pugh CW, Ratcliffe PJ, and Mole DR (2009) Regulation of growth differentiation factor 15 expression by intracellular iron. *Blood* **113:**1555–1563.

Le NT and Richardson DR (2004) Iron chelators with high antiproliferative activity up-regulate the expression of a growth inhibitory and metastasis suppressor gene: a link between iron metabolism and proliferation. *Blood* **104:**2967–2975.

Lee H and Paik SG (2006) Regulation of BNIP3 in normal and cancer cells. *Mol Cells* **21:**1–6.

Liang SX and Richardson DR (2003) The effect of potent iron chelators on the regulation of p53: examination of the expression, localization and DNA-binding activity of p53 and the transactivation of WAF1. *Carcinogenesis* **24:**1601–1614.

Liu J, Grogan L, Nau MM, Allegra CJ, Chu E, and Wright JJ (2001) Physical interaction between p53 and primary response gene Egr-1. *Int J Oncol* **18:**863–870.

Liu Y, Cox SR, Morita T, and Kourembanas S (1995) Hypoxia regulates vascular endothelial growth factor gene expression in endothelial cells. Identification of a 5′ enhancer. *Circ Res* **77:**638–643.

Lu X, Bocangel D, Nannenga B, Yamaguchi H, Appella E, and Donehower LA (2004) The p53-induced oncogenic phosphatase PPM1D interacts with uracil DNA glycosylase and suppresses base excision repair. *Mol Cell* **15:**621–634.

Lu X, Ma O, Nguyen TA, Jones SN, Oren M, and Donehower LA (2007) The Wip1 Phosphatase acts as a gatekeeper in the p53-Mdm2 autoregulatory loop. *Cancer Cell* **12:**342–354.

Lu X, Nannenga B, and Donehower LA (2005) PPM1D dephosphorylates Chk1 and p53 and abrogates cell cycle checkpoints. *Genes Dev* **19:**1162–1174.

Martinez JM, Sali T, Okazaki R, Anna C, Hollingshead M, Hose C, Monks A, Walker NJ, Baek SJ, and Eling TE (2006) Drug-induced expression of nonsteroidal anti-inflammatory drug-activated gene/macrophage inhibitory cytokine-1/prostate-derived factor, a putative tumor suppressor, inhibits tumor growth. *J Pharmacol Exp Ther* **318:**899–906.

May D, Itin A, Gal O, Kalinski H, Feinstein E, and Keshet E (2005) Ero1-L alpha plays a key role in a HIF-1-mediated pathway to improve disulfide bond formation and VEGF secretion under hypoxia: implication for cancer. *Oncogene* **24:**1011–1020.

Miyazaki K, Kawamoto T, Tanimoto K, Nishiyama M, Honda H, and Kato Y (2002) Identification of functional hypoxia response elements in the promoter region of the DEC1 and DEC2 genes. *J Biol Chem* **277:**47014–47021.

Morrish DW, Dakour J, and Li H (2001) Life and death in the placenta: new peptides and genes regulating human syncytiotrophoblast and extravillous cytotrophoblast lineage formation and renewal. *Curr Protein Pept Sci* **2:**245–259.

Muckenthaler M, Richter A, Gunkel N, Riedel D, Polycarpou-Schwarz M, Hentze S, Falkenhahn M, Stremmel W, Ansorge W, and Hentze MW (2003) Relationships

and distinctions in iron-regulatory networks responding to interrelated signals. *Blood* **101:**3690–3698.
Ozaki T, Li Y, Kikuchi H, Tomita T, Iwatsubo T, and Nakagawara A (2006) The intracellular domain of the amyloid precursor protein (AICD) enhances the p53-mediated apoptosis. *Biochem Biophys Res Commun* **351:**57–63.
Packer LM, Pavey SJ, Boyle GM, Stark MS, Ayub AL, Rizos H, and Hayward NK (2007) Gene expression profiling in melanoma identifies novel downstream effectors of p14ARF. *Int J Cancer* **121:**784–790.
Richardson D, Ponka P, and Baker E (1994) The effect of the iron(III) chelator, desferrioxamine, on iron and transferrin uptake by the human malignant melanoma cell. *Cancer Res* **54:**685–689.
Richardson DR and Bernhardt PV (1999) Crystal and molecular structure of 2-hydroxy-1-naphthaldehyde isonicotinoyl hydrazone (NIH) and its iron(III) complex: an iron chelator with antitumour activity. *J Biol Inorg Chem* **4:**266–273.
Richardson DR, Tran EH, and Ponka P (1995) The potential of iron chelators of the pyridoxal isonicotinoyl hydrazone class as effective antiproliferative agents. *Blood* **86:**4295–4306.
Rogers JT, Randall JD, Cahill CM, Eder PS, Huang X, Gunshin H, Leiter L, McPhee J, Sarang SS, Utsuki T, Greig NH, Lahiri DK, Tanzi RE, Bush AI, Giordano T, and Gullans SR (2002) An iron-responsive element type II in the 5′-untranslated region of the Alzheimer's amyloid precursor protein transcript. *J Biol Chem* **277:**45518–45528.
Rong Y, Hu F, Huang R, Mackman N, Horowitz JM, Jensen RL, Durden DL, Van Meir EG, and Brat DJ (2006) Early growth response gene-1 regulates hypoxia-induced expression of tissue factor in glioblastoma multiforme through hypoxia-inducible factor-1-independent mechanisms. *Cancer Res* **66:**7067–7074.
Saarikoski ST, Rivera SP, and Hankinson O (2002) Mitogen-inducible gene 6 (MIG-6), adipophilin and tuftelin are inducible by hypoxia. *FEBS Lett* **530:**186–190.
Sanchez M, Galy B, Muckenthaler MU, and Hentze MW (2007) Iron-regulatory proteins limit hypoxia-inducible factor-2alpha expression in iron deficiency. *Nat Struct Mol Biol* **14:**420–426.
Secchiero P, Barbarotto E, Tiribelli M, Zerbinati C, di Iasio MG, Gonelli A, Cavazzini F, Campioni D, Fanin R, Cuneo A, and Zauli G (2006) Functional integrity of the p53-mediated apoptotic pathway induced by the nongenotoxic agent nutlin-3 in B-cell chronic lymphocytic leukemia (B-CLL). *Blood* **107:**4122–4129.
Semenza GL (1999) Regulation of mammalian O2 homeostasis by hypoxia-inducible factor 1. *Annu Rev Cell Dev Biol* **15:**551–578.
Sevier CS and Kaiser CA (2008) Ero1 and redox homeostasis in the endoplasmic reticulum. *Biochim Biophys Acta* **1783:**549–556.
Sperandio S, Fortin J, Sasik R, Robitaille L, Corbeil J, and de Belle I (2009) The transcription factor Egr1 regulates the HIF-1alpha gene during hypoxia. *Mol Carcinog* **48:**38–44.
Stein S, Thomas EK, Herzog B, Westfall MD, Rocheleau JV, Jackson RS, 2nd, Wang M, and Liang P. NDRG1 is necessary for p53-dependent apoptosis. *J Biol Chem* 279(47):48930–48940, 2004.
Suryo Rahmanto Y, Dunn LL, and Richardson DR (2007) Identification of distinct changes in gene expression after modulation of melanoma tumor antigen p97 (melanotransferrin) in multiple models in vitro and in vivo. *Carcinogenesis* **28:**2172–2183.
Tai TC, Wong-Faull DC, Claycomb R, and Wong DL (2009) Hypoxic stress-induced changes in adrenergic function: role of HIF1 alpha. *J Neurochem* **109:**513–524.
Takayama K, Tsutsumi S, Suzuki T, Horie-Inoue K, Ikeda K, Kaneshiro K, Fujimura T, Kumagai J, Urano T, Sakaki Y, Shirahige K, Sasano H, Takahashi S, Kitamura T, Ouchi Y, Aburatani H, and Inoue S (2009) Amyloid precursor protein is a primary androgen target gene that promotes prostate cancer growth. *Cancer Res* **69:**137–142.
Togashi A, Katagiri T, Ashida S, Fujioka T, Maruyama O, Wakumoto Y, Sakamoto Y, Fujime M, Kawachi Y, Shuin T, and Nakamura Y (2005) Hypoxia-inducible protein 2 (HIG2), a novel diagnostic marker for renal cell carcinoma and potential target for molecular therapy. *Cancer Res* **65:**4817–4826.
van den Beucken T, Magagnin MG, Savelkouls K, Lambin P, Koritzinsky M, and Wouters BG (2007) Regulation of Cited2 expression provides a functional link between translational and transcriptional responses during hypoxia. *Radiother Oncol* **83:**346–352.
Wang R, Zhang YW, Zhang X, Liu R, Zhang X, Hong S, Xia K, Xia J, Zhang Z, and Xu H (2006) Transcriptional regulation of APH-1A and increased gamma-secretase cleavage of APP and Notch by HIF-1 and hypoxia. *FASEB J* **20:**1275–1277.
Whitnall M, Howard J, Ponka P, and Richardson DR (2006) A class of iron chelators with a wide spectrum of potent antitumor activity that overcomes resistance to chemotherapeutics. *Proc Natl Acad Sci U S A* **103:**14901–14906.
Yamada K and Miyamoto K (2005) Basic helix-loop-helix transcription factors, BHLHB2 and BHLHB3; their gene expressions are regulated by multiple extracellular stimuli. *Front Biosci* **10:**3151–3171.
Yan SF, Lu J, Zou YS, Soh-Won J, Cohen DM, Buttrick PM, Cooper DR, Steinberg SF, Mackman N, Pinsky DJ, and Stern DM (1999) Hypoxia-associated induction of early growth response-1 gene expression. *J Biol Chem* **274:**15030–15040.
Yang G, Zhang G, Pittelkow MR, Ramoni M, and Tsao H (2006) Expression profiling of UVB response in melanocytes identifies a set of p53-target genes. *J Invest Dermatol* **126:**2490–2506.
Yoshioka H, Kamitani H, Watanabe T, and Eling TE (2008) Nonsteroidal anti-inflammatory drug-activated gene (NAG-1/GDF15) expression is increased by the histone deacetylase inhibitor trichostatin A. *J Biol Chem* **283:**33129–33137.
Yu Y, Kalinowski DS, Kovacevic Z, Siafakas AR, Jansson PJ, Stefani C, Lovejoy DB, Sharpe PC, Bernhardt PV, and Richardson DR (2009) Thiosemicarbazones from the old to new: iron chelators that are more than just ribonucleotide reductase inhibitors. *J Med Chem* **52:**5271–5294.
Yuan J, Lovejoy DB, and Richardson DR (2004) Novel di-2-pyridyl-derived iron chelators with marked and selective antitumor activity: in vitro and in vivo assessment. *Blood* **104:**1450–1458.
Zagurovskaya M, Shareef MM, Das A, Reeves A, Gupta S, Sudol M, Bedford MT, Prichard J, Mohiuddin M, and Ahmed MM (2009) EGR-1 forms a complex with YAP-1 and upregulates Bax expression in irradiated prostate carcinoma cells. *Oncogene* **28:**1121–1131.
Zhang F, Wang W, Tsuji Y, Torti SV, and Torti FM (2008) Post-transcriptional modulation of iron homeostasis during p53-dependent growth arrest. *J Biol Chem* **283:**33911–33918.
Zhang X, Zhou K, Wang R, Cui J, Lipton SA, Liao FF, Xu H, and Zhang YW (2007) Hypoxia-inducible factor 1alpha (HIF-1alpha)-mediated hypoxia increases BACE1 expression and beta-amyloid generation. *J Biol Chem* **282:**10873–10880.
Zhou D, Chen B, Ye JJ, and Chen S (2004) A novel crosstalk mechanism between nuclear receptor-mediated and growth factor/Ras-mediated pathways through PNRC-Grb2 interaction. *Oncogene* **23:**5394–5404.

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