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Iron Chelation by Clinically Relevant Anthracyclines: Alteration in Expression of Iron-Regulated Genes and Atypical Changes in Intracellular Iron Distribution and Trafficking

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Running Title: Iron chelation and anthracyclines

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Abbreviations: 5-i-DAU, 5-imino-daunorubicin; DAU, daunorubicin; DFO, desferrioxamine; DOX, doxorubicin; EPI, epirubicin; FAC, ferric ammonium citrate; ferritin-H, ferritin heavy chain; ferritin-L, ferritin light chain; FPLC, fast pressure liquid chromatography; HIF-1α, hypoxia inducible factor-1α; HRE, hypoxia response element; IREs, iron-responsive element, IRP, iron regulatory protein; MEF, murine embryo fibroblast; MnTBAP, Mn(III) tetrakis (4-benzoic acid)-porphyrin; Ndrg1, N-myc downstream-regulated gene-1; PAGE, polyacrylamide gel electrophoresis; PIH, pyridoxal isonicotinoyl hydrazone; ROS, reactive oxygen species; RS, radical scavengers; SOD, superoxide dismutase; Tf, transferrin; TfR1, transferrin receptor-1; VEGF1, vascular endothelial growth factor-1.

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Abstract

Anthracyclines are effective anti-cancer agents. However, their use is limited by cardiotoxicity, an effect linked to their ability to chelate iron (Fe) and perturb Fe metabolism (Xu X, Persson L and Richardson DR *Mol Pharmacol* 68:261-271, 2005). These effects on Fe-trafficking remain poorly understood, but are important to decipher as treatment for anthracycline cardiotoxicity utilizes the chelator, dexrazoxane. Incubation of cells with doxorubicin (DOX) up-regulated mRNA levels of the Fe-regulated genes, *transferrin receptor-1* (*TfR1)* and *N-myc downstream-regulated gene-1* (*Ndrg1*). This effect was mediated by Fe-depletion, as it was reversed by adding Fe and was prevented by saturating the anthracycline metal-binding site with Fe. However, DOX did not act like a typical chelator, as it did not induce cellular Fe mobilization. In the presence of DOX and ^{59}Fe-transferrin, Fe-trafficking studies demonstrated ferritin-^{59}Fe accumulation and decreased cytosolic-^{59}Fe incorporation. This could induce cytosolic Fe-deficiency and increase *TfR1* and *Ndrg1* mRNA. Up-regulation of *TfR1* and *Ndrg1* by DOX was independent of anthracycline-mediated radical generation and occurred *via* HIF-1α-independent mechanisms. Despite increased *TfR1* and *Ndrg1* mRNA after DOX treatment, this agent decreased TfR1 and Ndrg1 protein expression. Hence, the effects of DOX on Fe metabolism were complex due to its multiple effector mechanisms.

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Introduction

Anthracyclines are known iron (Fe) chelators (Figure 1A), but their effects on cellular Fe metabolism are poorly understood (Xu et al., 2005). These compounds have high activity against hematological malignancies and a variety of other tumors (Xu et al., 2005). However, a major problem is their cardiotoxic effect at high cumulative doses that limit their clinical use (Gianni and Myers, 1992). The mechanism of anthracycline-mediated cardiotoxicity is unclear (Kaiserova et al., 2007), probably because of the multiple effects of these agents, including DNA-binding, intercalation, alkylation, inhibition of topoisomerase II and the generation of reactive oxygen species (ROS) (Gianni and Myers, 1992).

Previous studies have indicated that interactions of anthracyclines with cellular Fe pools are of great importance in their cardiotoxic effects and ability to induce apoptosis (Hershko et al., 1993; Kotamraju et al., 2002). Anthracyclines such as doxorubicin (DOX) can directly chelate Fe(III) forming an Fe complex with an overall association constant of 10^{33}(Beraldo et al., 1985; May et al., 1980). Hershko and associates demonstrated that Fe-loading potentiates the cardiotoxic effects of anthracyclines (Hershko et al., 1993; Link et al., 1996) and some chelators can prevent this (Kaiserova et al., 2007). In fact, the clinical intervention for anthracycline cardiotoxicity involves the chelator, dexrazoxane (Xu et al., 2005). Hence, understanding the mechanisms of how anthracyclines interfere with Fe metabolism is a key for preventing cardiotoxicity.

Iron is transported by its binding to transferrin (Tf) and is delivered to cells *via* binding to the transferrin receptor 1 (TfR1) (Xu et al., 2005). After this, Tf is internalized by receptor-mediated endocytosis and the Fe is released. Iron is then transported into the cell and becomes part of the intracellular Fe pool. Iron that is not used for metabolic requirements is stored in ferritin, a polymeric protein consisting of H- and L-subunits (Minotti et al., 2004a).

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The translation of TfR1 and ferritin are regulated by the binding of iron regulatory proteins (IRPs) to iron-responsive elements (IREs) present in the 5'- or 3'-untranslated regions of *TfR1* and *ferritin* mRNAs (Xu et al., 2005). There are two IRPs, IRP1 and IRP2, and anthracyclines have been shown to decrease their mRNA-binding activity in most cell types (Kwok and Richardson, 2002; Minotti et al., 2001).

Apart from the effect of anthracyclines on IRP-mRNA-binding activity, these agents have been shown to affect a variety of molecules and metabolic pathways involved in Fe metabolism (Minotti et al., 2004a). For instance, DOX is known to directly bind Fe and has been reported to remove Fe from isolated ferritin, Tf and microsomal membranes (Xu et al., 2005). However, using intact cells, we showed that incubation of many cell types with anthracyclines (Figure 1A) such as doxorubicin (DOX), daunorubicin (DAU) or epirubicin (EPI) induced ferritin Fe-loading, due to their ability to prevent Fe release from this protein (Kwok and Richardson, 2003; Kwok and Richardson, 2004). The precise mechanism by which anthracyclines prevent ferritin-Fe mobilization was not clear, but inhibition of protein synthesis and/or proteasomal/lysosomal activity were suggested to be involved (Kwok and Richardson, 2004). Incubation of cells with DOX also increased ferritin expression (Corna et al., 2004; Kwok and Richardson, 2003) and this was suggested to act as a protective response against the ability of DOX to generate ROS (Corna et al., 2004).

In the present study, we demonstrate for the first time that anthracyclines act as atypical chelators, having a number of effects on cellular Fe metabolism and the expression of Fe-regulated genes, including *TfR1*, *Ndrg1* and *ferritin*. While Fe chelation mediated by anthracyclines increased *TfR1* and *Ndrg1* mRNA expression, the protein levels of these molecules were decreased. Paradoxically, ferritin protein expression increased after

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incubation with DOX as did ferritin Fe accumulation, suggesting that anthracyclines have a selective effect on gene expression. The effects of anthracyclines on cellular Fe metabolism were complex, probably since they act on multiple molecular targets.

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Materials and Methods

Reagents

Desferrioxamine (DFO) was from Novartis (Basel, Switzerland). DOX, DAU and EPI were from Pharmacia (Sydney, Australia). All other reagents were from Sigma-Aldrich (St. Louis, MO). Pyridoxal isonicotinoyl hydrazone (PIH) was synthesized and characterized by standard methods (Ponka et al., 1979; Richardson et al., 1995).

Cell Culture

Cell lines were obtained from the American Type Culture Collection (Manassas, VA). Murine embryonic fibroblasts (MEFs) from wild-type and homozygous *hypoxia inducible factor-1α* (*HIF-1α*) knockout mice were obtained from Dr. R. Johnson (University of California, San Diego, USA). Cells were grown using standard conditions (Kwok and Richardson, 2003; Le and Richardson, 2004).

^{59}Fe-Transferrin

Human apotransferrin (apo-Tf; Sigma-Aldrich) was labeled with ^{59}Fe (Dupont NEN, MA) to produce $^{59}Fe_2$-transferrin (^{59}Fe-Tf), as described (Kwok and Richardson, 2003). Briefly, apo-Tf was labeled with Fe using the ferric-nitrilotriacetate complex at a ratio of 1 Fe to 10 NTA. This complex was prepared in 0.1 M HCl and then this solution adjusted to pH 7.4 using 1.4% $NaHCO_3$. This solution was added to apo-Tf and then incubated for 1 h at 37^{o}C. Unbound Fe was removed by exhaustive vacuum dialysis against 0.15 M NaCl adjusted to pH 7.4 using 1.4% $NaHCO_3$. The saturation of Tf with Fe was monitored by UV-Vis spectrophotometry with the absorbance at 280 nm (protein) being compared with that at 465 nm (Fe-binding site). In all studies, fully saturated diferric Tf was used.

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Effect of Anthracyclines on ^{59}Fe Efflux from Intact Prelabeled Cells

Experiments examining the ability of agents to mobilize cellular-^{59}Fe were performed using standard techniques (Richardson et al., 1995). Briefly, cells were prelabeled with ^{59}Fe-Tf (0.75 μM) for 3 h at 37^{o}C. This medium was aspirated and the cell monolayer washed 4 times with ice-cold PBS. The cells were then reincubated for 24 h at 37^{o}C with medium in the presence or absence of the agents to be tested. After this incubation, the overlying media containing released ^{59}Fe were collected in γ-counting tubes. The cells were removed from the petri dishes and placed in a separate set of tubes. Radioactivity was measured in both the cell pellet and supernatant using a Wallac Wizard 1480 3" γ-counter (Turku, Finland).

Assay for Examining the Ability of Anthracyclines to Bind ^{59}Fe from Cell Lysates

Established methods (Watts and Richardson, 2002) using ultrafiltration through a 5-kDa cut-off filter were used to determine the efficacy of anthracyclines at mobilizing ^{59}Fe from SK-Mel-28 cell lysates. Briefly, cells were labeled with ^{59}Fe-Tf (0.75 μM) for 3 h at 37^{o}C, placed on a tray of ice, the medium decanted and the cell monolayer washed four times with ice-cold PBS. The cells were lysed by one freeze-thaw cycle and then detached from the flask using a Teflon spatula in the presence of the non-ionic detergent Triton X-100 (1.5%) at 4^{o}C. The supernatant was obtained by centrifugation at 16,500 x*g* for 30 min at 4^{o}C and then incubated for 3 h at 37^{o}C with the agents of interest. After this incubation, the samples were then subjected to centrifugation at 4^{o}C through a 5-kDa M_r exclusion filter (Millipore, Billerica, MA). After centrifugation, the eluate was taken to estimate ^{59}Fe levels.

Determination of Intracellular Iron Distribution using Native-PAGE-^{59}Fe-Autoradiography

Native-gradient-PAGE-^{59}Fe-autoradiography was performed using established techniques (Babusiak et al., 2005). Briefly, cells labeled with ^{59}Fe-Tf (0.75 μM) were lysed at 4^{o}C in

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buffer containing 1.5% Triton X-100, 0.14 M NaCl and 20 mM HEPES (pH 8) supplemented with an EDTA-free protease inhibitor cocktail (Roche, Penzberg, Germany). Samples were then vortexed and centrifuged at 16,000 x*g* for 45 min at 4°C. The supernatants were loaded onto a native 3-12% gradient PAGE gel (100 µg protein/lane) and electrophoresis was performed at 20 mA/gel overnight at 4°C. Gels were subsequently dried and autoradiography was performed. Bands on X-ray film were quantified by scanning densitometry and analyzed using the program, Quantity One (Bio-Rad, Hercules, CA).

Fast Pressure Liquid Chromatography (FPLC) and Native Gradient PAGE

SK-Mel-28 cells were incubated with or without DOX (2 µM) in the presence of ^{59}Fe-Tf (0.75 µM). Cells were then washed 4-times and lysed on ice in 20 mM HEPES/140 mM NaCl/1.5% Triton X-100 (pH 8). Cell lysates were centrifuged at 16,500 x*g* and the supernatant was loaded onto a Superdex 200 10/300 GL column (GE Healthcare, Bucks, UK) and proteins eluted with 20 mM HEPES/140 mM NaCl (pH 8) using FPLC (Bio-Rad, Hercules, CA). Fractions (1 mL) were collected and radioactivity examined using the γ-counter above.

Fractions were concentrated and desalted using the microfilter units described above with a 5 kDa molecular mass (M_r) cut-off. Concentrated fractions were then separated and examined *via* native-gradient-PAGE-^{59}Fe-autoradiography (Babusiak et al., 2005).

RNA Isolation, RT-PCR and Western Analysis

RNA isolation and reverse-transcriptase PCR (RT-PCR) were performed by published procedures (Le and Richardson, 2004) using the primers in Table I. Western blot analysis was done as described (Le and Richardson, 2004).

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^{3}H-Leucine Incorporation Assay

To assess protein synthesis, ^{3}H-leucine assays were performed using standard procedures (Kwok and Richardson, 2004).

Statistical Analysis.

Data were compared using the Student's *t*-test. Results were considered statistically significant when $p < 0.05$.

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Results

Challenge of DOX-Treated Cells with Iron Leads to Decreased Viability

Incubation of cells with anthracyclines leads to alterations in Fe metabolism (Corna et al., 2004; Kwok and Richardson, 2003; Xu et al., 2005). Initial experiments examined if DOX altered the ability of cells to protect against a challenge with excess Fe. In these studies, SK-Mel-28 melanoma cells or H9c2 cardiomyocytes were preincubated for 24 h at 37^{o}C with DOX (5 μM) and then reincubated for 16 h at 37^{o}C with increasing concentrations of ferric ammonium citrate (FAC; 250-1000 μg/mL), that donates Fe to cells (Corna et al., 2004). Direct cell counts and viability were then assessed using Trypan blue staining. These incubation conditions were identical to those used by others to demonstrate the protective effect against an Fe challenge of preincubating H9c2 cells with DOX (Corna et al., 2004).

In contrast to results by others (Corna et al., 2004), preincubation with DOX did not protect against an Fe challenge. In fact, it resulted in significantly decreased viability of H9c2 and SK-Mel-28 cells at FAC concentrations >500 μg/mL (Figure 1B). Hence, DOX decreased the ability of cells to appropriately accomodate the Fe load and prevent its cytotoxic effects.

DOX Increases mRNA Expression of the Fe-Responsive Genes, TfR1 and Ndrg1

To further understand how DOX affects Fe metabolism, we investigated the effect of DOX on *TfR1* expression (Figure 2). To examine this, SK-Mel-28 cells were initially used (Figure 2A) as their Fe metabolism is well characterized and these cells were previously utilized to assess the effects of DOX on Fe trafficking (Kwok and Richardson, 2002; Kwok and Richardson, 2003; Kwok and Richardson, 2004).

Incubation of cells for 24 h at 37^{o}C with the Fe chelator, desferrioxamine (DFO; 100 μM), was used as a positive control as it increases *TfR1* mRNA and protein expression (Hentze and

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Kuhn, 1996). Incubation of SK-Mel-28 cells with DFO increased *TfR1* expression >6-fold compared to the control (Figure 2A). DOX (0.5-5 μM) induced a dose-dependent increase in *TfR1* mRNA up to 2 μM where its expression was 3-fold greater than the control (Figure 2A). The up-regulation of *TfR1* mRNA after incubation with DOX was relatively marked considering the dose maximally up-regulating its expression (2 μM) was 50-fold lower than DFO (100 μM; Figure 2A). At 5 μM DOX, *TfR1* expression then decreased and this down-regulation may be related to the drug acting as a transcriptional inhibitor (Tarr and van Helden, 1990).

The increase in *TfR1* mRNA after incubation of SK-Mel-28 cells with DOX may be mediated *via* its ability to act as an Fe chelator (Gianni and Myers, 1992; May et al., 1980). Examination of four other cell types also demonstrated that DOX increased *TfR1* mRNA, although the dose-dependence and extent of up-regulation was different for each cell type (Figure 2B-E). Generally, maximum *TfR1* mRNA expression was found at 1-2 μM DOX.

Typically, Fe chelation is known to up-regulate *TfR1* mRNA by the IRP-IRE mechanism (Hentze and Kuhn, 1996). However, the lower DOX concentrations (1-2 μM) used in this study have little effect on IRP-mRNA-binding activity in SK-Mel-28 cells (Kwok and Richardson, 2002). Thus, it was unclear if this mechanism was responsible for DOX-mediated up-regulation of *TfR1* mRNA (Figure 2A). Apart from the IRPs, other Fe-sensing mechanisms could be responsible for alterating *TfR1* mRNA expression. Considering this, HIF-1α protein expression is known to increase after Fe chelation or hypoxia and can transcriptionally up-regulate *TfR1* and other genes (Beerepoot et al., 1996; Bianchi et al., 1999; Le and Richardson, 2004; Lok and Ponka, 1999).

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To determine if HIF-1α activity is affected by anthracyclines, we examined the effect of DOX on HIF-1α target gene expression. These studies investigated the metastasis suppressor, *N-myc downstream regulated gene-1* (*Ndrg1*), that is known to be up-regulated after Fe chelation by HIF-1α (Le and Richardson, 2004). This gene was important to assess as its anti-proliferative and -metastatic effects could be relevant to DOX activity (Kovacevic and Richardson, 2006). Similarly to *TfR1*, DOX also increased *Ndrg1* mRNA expression in SK-Mel-28 cells (Figure 2A). Assessment of 4 other cell types demonstrated that as for SK-Mel-28 cells, DFO increased *Ndrg1* mRNA (Figure 2B-E). Again, the response of *Ndrg1* mRNA levels to increasing DOX concentrations was variable in terms of dose-response and the extent of up-regulation between cell types (Figure 2A-E). The differences in gene expression between these cell types may relate to variation in the uptake and metabolism of DOX. Of interest, the mRNA levels of another HIF-1α target gene, namely *Nip3* (Bruick, 2000), was also up-regulated by DOX in a similar way to *TfR1* and *Ndrg1* (data not shown). Further studies then examined the effect of other anthracyclines on Fe metabolism using the SK-Mel-28 cell type.

Daunorubicin and Epirubicin also Increase TfR1 and Ndrg1 mRNA Expression

DAU and EPI are structurally-related to DOX (Figure 1A) and also increased *TfR1* and *Ndrg1* mRNA in a dose-dependent manner (Figure 3A). However, the response of SK-Mel-28 melanoma cells to each of the anthracyclines was different (Figure 3A). Daunorubicin gradually increased *TfR1* and *Ndrg1* mRNA up to 5 μM, with the effect at this latter concentration being similar to 2 μM DOX (Figure 3A). As found for DOX, EPI increased *TfR1* and *Ndrg1* mRNA up to 2 μM and then at the highest EPI concentration assessed (*i.e.*, 5 μM), the expression of these genes decreased (Figure 3A). Generally, these results demonstrated that DOX, DAU and EPI increased *TfR1* and *Ndrg1* mRNA up to a concentration of 1-2 μM.

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Anthracyclines Increase TfR1 and Ndrg1 Expression as a Function of Time

The effect of anthracyclines on *TfR1* and *Ndrg1* mRNA was then assessed as a function of incubation time. The optimal anthracycline concentration that up-regulated gene expression in SK-Mel-28, namely 2 μM (Figure 2A), was incubated with this cell type for 3-24 h at 37^{o}C. The effect of DOX was compared to the positive control, DFO (100 μM). In these studies, DFO increased *TfR1* and *Ndrg1* mRNA expression after 6 h (Figure 3B). This was in agreement with previous studies using DFO and other cell types (Le and Richardson, 2004). A significant ($p < 0.05$) increase in *TfR1* mRNA expression after incubation with the anthracyclines was evident after 18 h. However, the anthracyclines increased *Ndrg1* mRNA expression after only 6 h to a comparable or greater extent than DFO (Figure 3B).

The DOX-Mediated Increase in TfR1 and Ndrg1 mRNA is Iron-Dependent

DOX, DAU and EPI possess the same Fe-binding sites (carbonyl and hydroxyl moieties) that are necessary for Fe chelation (Figure 1A). The ability of these agents to bind Fe was shown in the "test tube" (May et al., 1980), but not in intact cells. Certainly, these compounds may be acting as chelators to deplete Fe pools and increase *TfR1* and *Ndrg1* mRNA. To examine this, the effect of DFO (2 μM) was compared to the anthracyclines at the same concentration. Furthermore, the efficacy of the anthracyclines at increasing *Ndrg1* and *TfR1* was also compared to their pre-formed 3:1 ligand-Fe(III) complexes (Figure 4A).

After a 24 h incubation, DFO clearly increased *TfR1* and *Ndrg1* mRNA expression (Figure 4A). The 1:1 DFO-Fe complex largely prevented *TfR1* and *Ndrg1* up-regulation that was observed with DFO. The anthracyclines, DOX, DAU and EPI all increased both *TfR1* and *Ndrg1* mRNA expression, while their Fe complexes were significantly ($p < 0.001$) less effective over 3 experiments. Hence, this suggested that up-regulation of *TfR1* and *Ndrg1* was

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due to anthracyclines binding cellular Fe (Figure 4A). Interestingly, the effect of the anthracyclines at inducing *Ndrg1* expression was more pronounced than that observed with *TfR1* (Figure 4A).

Further experiments assessed whether DOX-mediated up-regulation of *TfR1* and *Ndrg1* could be reversed by Fe added as FAC (100 μg/mL; Figure 4B, C). SK-Mel-28 cells were pre-incubated with control medium (CON), DFO (100 μM) or DOX (2 μM) for 20 h (primary incubation), and then re-incubated for another 20 h (secondary incubation) with CON, FAC (100 μg/mL), DFO (100 μM) or DOX (2 μM).

After primary incubation with CON, secondary incubation with FAC (Figure 4B, C; lane 2) decreased *TfR1* and *Ndrg1* mRNA levels compared to cells treated with CON (Figure 4B, C; lane 1). The treatment with FAC acted as a positive control to demonstrate both genes are Fe-regulated. Cells treated with DFO or DOX followed by CON (Figure 4B, C; lane 4, 7) led to increased *TfR1* and *Ndrg1* mRNA expression compared to the control (Figure 4B, C; lane 1). Depletion of cellular Fe by primary and secondary incubation with DFO resulted in more pronounced up-regulation of *TfR1* and *Ndrg1* levels (Figure 4B, C; lane 6) in comparison to DFO followed by CON (Figure 4B; lane 4). Primary and secondary incubation with DOX caused similar up-regulation of *TfR1* and *Ndrg1* (Figure 4B, C; lane 9) as DOX followed by CON (Figure 4B, C; lane 7). Importantly, primary incubation with DFO or DOX and reincubation with FAC (Figure 4B, C; lanes 5 and 8) significantly ($p < 0.01$) decreased *TfR1* and *Ndrg1* up-regulation compared to the relative control (Figure 4B, C; lanes 4 and 7). This further confirmed that anthracyclines increased *TfR1* and *Ndrg1* mRNA *via* Fe chelation and this up-regulation was reversible upon adding Fe.

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DOX Does Not Induce Cellular Fe Mobilization but Causes Intracellular Fe Re-distribution

To understand how DOX affected Fe metabolism to up-regulate *TfR1* and *Ndrg1* mRNA, studies examined its effects on cellular ^{59}Fe mobilization. The ability of DOX (0.1-5 μM) at mobilizing ^{59}Fe was compared to the chelators, DFO (100 μM) and PIH (25 μM), over 24 h at 37^{o}C (Figure 5A). These DOX concentrations were chosen as they were used to examine *TfR1* and *Ndrg1* mRNA expression (Figures 2-4). Both DFO and PIH increased cellular-^{59}Fe mobilization to 225 and 270% of the control, while DOX had no effect (Figure 5A). Further studies examined the ability of DOX to mobilize ^{59}Fe from cell lysates. In contrast to the positive control, DFO (100 μM), which caused marked ^{59}Fe mobilization from lysates, DOX (0.5-5 μM) had no effect (Figure 5B). Collectively, despite DOX having high Fe-binding affinity (May et al., 1980) and its ability to up-regulate *TfR1* and *Ndrg1* mRNA by Fe-depletion (Figure 4), it does not act like a typical chelator to induce Fe efflux.

Further studies were performed using FPLC to examine alterations in intracellular ^{59}Fe distribution (Figure 5C). Cells were labeled with ^{59}Fe-Tf (0.75 μM) in the presence or absence of DOX (2 μM) for 24 h at 37^{o}C, then washed, lysed and centrifuged. The supernatant was fractionated on a size-exclusion column and the fractions measured for ^{59}Fe. In control cells, two major high M_r peaks were detected (Figure 5C). According to the column calibration, the first peak at fraction 12 (F12) represented ^{59}Fe-containing molecules of ≈ 700 kDa. A second peak at fraction 15 (F15) co-migrated with horse spleen ferritin (≈ 400 kDa; Figure 5C). After incubation with DOX, ^{59}Fe in F12 was significantly ($p < 0.01$) decreased over 3 experiments. In contrast, in the ferritin fraction (F15) there was a significant increase in ^{59}Fe incorporation. There were two other lower M_r peaks eluting at fractions 20 and 27, although there was no significant difference between them comparing control and DOX-treated cells (Figure 5C).

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To further elucidate the nature of the ^{59}Fe-containing molecules, F12 and F15 were concentrated and separated using native-gradient PAGE (Figure 5D). These studies showed that DOX decreased ^{59}Fe incorporation into high M_r proteins (F12), while there was ferritin-^{59}Fe accumulation (F15). The ferritin-^{59}Fe loading was confirmed by addition of anti-ferritin antibody to the latter fraction leading to a super-shifted ferritin band (Figure 5D). These data demonstrated re-distribution of ^{59}Fe between ferritin and other ^{59}Fe-containing proteins, extending our previous observations (Kwok and Richardson, 2003; Kwok and Richardson, 2004). This ferritin-^{59}Fe accumulation leads to cytosolic Fe-deficiency that may up-regulate *TfR1* and *Ndrg1* mRNA (Figures 2, 3).

HIF-1α-Independent Mechanisms are Involved in Up-Regulation of TfR1 and Ndrg1 after Incubation with DOX.

The up-regulation of *TfR1* mRNA by anthracyclines could occur by the classical IRP mechanism (Hentze and Kuhn, 1996) and/or also *via* HIF-1α since the *TfR1* promoter contains a hypoxia response element (HRE; (Bianchi et al., 1999; Lok and Ponka, 1999). The increase in *Ndrg1* mRNA expression after Fe chelation by DFO was previously shown to occur by HIF-1α-dependent and -independent mechanisms (Le and Richardson, 2004).

To examine the role of HIF-1α in *TfR1* and *Ndrg1* up-regulation after incubation with DOX, we utilized *HIF-1α* knockout (*HIF-1α*$^{-/-}$) MEFs in comparison to wild-type (*HIF-1α*$^{+/+}$) MEFs (Ryan HE, 2000) (Figure 6A). Both *HIF-1α*$^{+/+}$ and *HIF-1α*$^{-/-}$ MEFs were incubated with DFO (100 μM; positive control) or DOX (2 μM) for 8 h and then *TfR1*, *Ndrg1* and *HIF-1α* mRNA expression was assessed (Figure 6A). Incubation of *HIF-1α*$^{+/+}$ or *HIF-1α*$^{-/-}$ cells with DFO or DOX increased *TfR1* mRNA levels irrespective of *HIF-1α* status, suggesting another mechanism was responsible. For DFO, this could be mediated by the IRPs (Hentze and Kuhn, 1996). Previous studies examining SK-Mel-28 cells demonstrated that at high

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DOX concentrations (*ie.*, 20 μM), IRP-mRNA-binding activity was reduced (Kwok and Richardson, 2002). However, at low concentrations (1 μM), IRP-binding was not markedly affected (Kwok and Richardson, 2002). This suggested the DOX-induced *TfR1* mRNA up-regulation at 1-2 μM in SK-Mel-28 cells (Figure 2A) may not be mediated by IRPs.

The expression of *Ndrg1* mRNA was more significantly up-regulated ($p < 0.05$) by DFO in *HIF-1α*$^{+/+}$ cells than their *HIF-1α*$^{-/-}$ counterparts (Figure 6A), in agreement with previous studies (Le and Richardson, 2004). This suggests that HIF-1α is important in up-regulating *Ndrg1* mRNA after Fe chelation, but that a HIF-1α-independent mechanism was also present (Le and Richardson, 2004). The up-regulation of *Ndrg1* mRNA after incubation of DOX occurred in *HIF-1α*$^{-/-}$ and *HIF-1α*$^{+/+}$ cells (Figure 6A), suggesting the response was HIF-1α-independent. In fact, in 3 experiments, *Ndrg1* mRNA up-regulation was significantly ($p < 0.045$) more marked in *HIF-1α*$^{-/-}$ than *HIF-1α*$^{+/+}$ cells (Figure 6A).

The effect of DOX and DFO was also examined on the expression of *vascular endothelial growth factor-1* (*VEGF1*) mRNA (Figure 6A) which is a typical HIF-1α-regulated gene (Beerepoot et al., 1996). The ability of DFO at increasing *VEGF1* mRNA was more pronounced in *HIF-1α*$^{+/+}$ than *HIF-1α*$^{-/-}$ cells. Hence, similarly to *Ndrg1*, this indicates HIF-1α is important in up-regulating *VEGF1* mRNA after DFO, but that a HIF-1α-independent mechanism was also present. After incubation with DOX, *VEGF1* mRNA was more highly expressed in *HIF-1α*$^{-/-}$ cells than *HIF-1α*$^{+/+}$ cells, indicating the anthracycline was up-regulating this gene *via* a HIF-1α-independent mechanism.

As an appropriate control, HIF-1α status was examined in *HIF-1α*$^{+/+}$ and *HIF-1α*$^{-/-}$ cell types. In these studies, *HIF-1α* mRNA expression was clearly evident in *HIF-1α*$^{+/+}$ cells and not

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markedly affected by the incubation with DFO or DOX. In contrast, and as expected, no transcript was detected in *HIF-1α*$^{-/-}$ cells (Figure 6A).

Activity of Free Radical Scavengers on Ndrg1 and TfR1 Expression after Incubation with Anthracyclines

Anthracyclines are well known to generate radicals (Corna et al., 2004) and increased TfR1 protein expression occurs after oxidant stress, at least in part, through IRP activation (Pantopoulos and Hentze, 1995). To determine the role of anthracycline-induced oxidant stress in *TfR1* and *Ndrg1* mRNA expression, we assessed the effect of radical scavengers (RS) on DOX-induced *TfR1* and *Ndrg1* mRNA expression (Figure 6B) and also the ability of DOX to inhibit protein synthesis (Figure 6C). In these experiments, we combined superoxide dismutase (SOD; 1000 U/mL) and catalase (1000 U/mL) with the cell-permeable glutathione peroxidase mimetic ebselen (15 μM) and cell-permeable SOD mimetic, MnTBAP (200 μM), as these agents alone and in combination are effective RS (Kotamraju et al., 2002; Kwok and Richardson, 2002). The addition of the RS had no significant effect on the up-regulation of either *TfR1* or *Ndrg1* mRNA by either DOX or DFO over 3 experiments (Figure 6B). This suggested *TfR1* and *Ndrg1* mRNA up-regulation was not due to anthracycline-induced oxidant stress.

As a positive control to demonstrate that RS reduced ROS generation and the cytotoxic effects of anthracyclines, experiments were performed with various anthracyclines to assess their ability to inhibit protein synthesis (^{3}H-leucine incorporation) in the presence and absence of the same combination of RS (Figure 6C). In these studies, DOX and DAU were compared to 5-imino-daunorubicin (5-i-DAU), that generates less ROS than the former anthracyclines (Corna et al., 2004).

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All anthracyclines were effective at reducing ^{3}H-leucine incorporation (Figure 6C). From the anthracyclines examined, DOX was the most effective, while 5-i-DAU demonstrated the least ability to inhibit ^{3}H-leucine incorporation (Figure 6C). This could be because 5-i-DAU is less redox active than DOX (Corna et al., 2004). For all anthracyclines, the combination with RS significantly ($p < 0.05$) increased ^{3}H-leucine incorporation compared to their relative controls (Figure 6C). Hence, the RS could partially rescue the effects of anthracyclines at depressing ^{3}H-leucine incorporation.

DOX Inhibits the Translation of TfR1 and Ndrg1 mRNA into Protein, while Ferritin Protein Expression Increases

The ability of DOX to prevent ^{3}H-leucine incorporation into protein suggested mRNA translation could be inhibited. These data agree with our earlier studies using SK-Mel-28 cells where DOX markedly inhibited ^{3}H-leucine incorporation (Kwok and Richardson, 2004). Hence, it was important to investigate if up-regulation of *TfR1* and *Ndrg1* mRNA after incubation with DOX (Figure 2 and 3) leads to increased protein expression.

At the lowest DOX concentration (0.5 μM), a slight but not significant increase in TfR1 protein expression occurred in SK-Mel-28 melanoma cells relative to the control (Figure 7A). At the same concentration, a more pronounced and significant ($p < 0.04$) increase in Ndrg1 protein expression was found relative to the control (Figure 7B). However, at higher DOX concentrations (5 and 7.5 μM), TfR1 and Ndrg1 protein expression decreased, potentially due to inhibition of protein synthesis (Figure 6C). In contrast, ferritin-H and -L protein levels increased in the presence of DOX (Figure 7C), in agreement with previous studies (Corna et al., 2004; Kwok and Richardson, 2003). It is also of interest that *ferritin –H* and *–L* mRNA increased as a function of DOX concentration up to 5 μM (Figure 7D), which is in contrast to

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TfR1 and *Ndrg1* mRNA which decreased at this latter concentration (Figure 2A). This indicated differential effects of DOX on gene expression.

Preincubation with DOX followed by Labeling with ^{59}Fe-Transferrin Decreases Cellular ^{59}Fe Uptake

Considering the decreased TfR1 protein expression at higher DOX concentrations (Figure 7A), studies were performed to examine the effect of DOX on ^{59}Fe uptake from ^{59}Fe-Tf (Figure 7E). After a 24 h preincubation with DOX (2 μM), cells were incubated with ^{59}Fe-Tf (0.75 μM) for 0.5-4 h. There was a significant ($p < 0.05$) decrease in ^{59}Fe uptake after 1-4 h in cells preincubated with DOX compared to control medium (Figure 7E). The intracellular distribution of ^{59}Fe was then assessed using native-gradient-PAGE-^{59}Fe-autoradiography (Babusiak et al., 2005) (Figure 7F). Again, cells were preincubated for 24 h at 37^{o}C with control medium or DOX (2 μM), washed and then incubated with ^{59}Fe-Tf (0.75 μM) for up to 4 h at 37^{o}C. Most ^{59}Fe was incorporated into a band in the middle of the gel which was shown to be ferritin by super-shift studies with an anti-ferritin antibody (Figure 7F; lanes 9 and 10). Transferrin migrated below ferritin as demonstrated using purified ^{59}Fe-Tf (Figure 7F; lane 11). The ferritin-^{59}Fe uptake was linear up to 4 h, with less ^{59}Fe being incorporated into cells preincubated with DOX.

Preincubation with DOX decreased both ^{59}Fe-Tf uptake (Figure 7E) and ^{59}Fe incorporation into ferritin (Figure 7F). This was in contrast to studies with no preincubation period, where DOX and ^{59}Fe-Tf were incubated together for 24 h, leading to ferritin-^{59}Fe accumulation (Figure 5C, D). Clearly, preincubation with DOX before the addition of ^{59}Fe-Tf inhibits protein synthesis (Figure 6C) which is a crucial secondary event that decreases TfR1 and thus ^{59}Fe uptake.

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Discussion

Anthracyclines bind Fe and act as bidentate chelators *via* their carbonyl and hydroxyl groups (Figure 1A) ((May et al., 1980). The same ligating sites are involved in Fe chelation by the effective chelator, deferiprone (Kalinowski and Richardson, 2005), and thus the effects of anthracyclines on Fe metabolism are important to dissect. However, the effects of anthracyclines on metabolism are complex since these agents have multiple molecular targets (Minotti et al., 2004a; Xu et al., 2005). In this study, we demonstrate for the first time that anthracyclines act as atypical chelators, having a number of effects on Fe metabolism and the expression of Fe-regulated genes.

Previous work suggested that preincubation with DOX protected cells from an Fe challenge due to increased ferritin expression (Corna et al., 2004). In this investigation, we repeated this experiment and demonstrated that preincubation with DOX followed by an Fe challenge did not protect cells. In fact, it was detrimental, resulting in decreased cellular viability (Figure 1B). The reason for this observation is probably related to several factors. First, it was shown by Link and colleagues (Link et al., 1996) that Fe-loading potentiates the cytotoxic effect of DOX, which is probably through the generation of a redox-active DOX-Fe complex (Gianni and Myers, 1992). Second, we previously demonstrated that incubation of cells with DOX prevented ferritin Fe release (Kwok and Richardson, 2004), which may be related to its ability to act as a protein synthesis inhibitor and/or inhibit lysosomal and proteasomal activity (Kwok and Richardson, 2004). The inability of ferritin to release Fe for essential metabolic processes would not be beneficial and could play a role in the cytotoxicity of anthracyclines. Third, in combination with the other well characterized cytotoxic effects of anthracyclines *e.g.*, inhibition of topoisomerase II, DNA intercalation *etc* (Minotti et al., 2004b), the multiple effects of preincubating cells with DOX markedly affects cellular metabolism, leading to an ineffective response to an Fe challenge.

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While chemical studies have shown that anthracyclines directly bind Fe (May et al., 1980), the intracellular consequences of this Fe-depletion have not been established. This is probably due to the complexity of their cellular interactions (Minotti et al., 2004a; Xu et al., 2005). In this study, we demonstrated that DOX, DAU and EPI could act like the well known chelator, DFO, increasing mRNA expression of the Fe-regulated genes, *TfR1* (Hentze and Kuhn, 1996) and *Ndrg1* (Le and Richardson, 2004). This effect was marked, as at an equimolar concentration to DFO (2 μM), all the anthracyclines were as, or more effective at increasing *TfR1* and *Ndrg1* mRNA (Figure 4A). The high Fe chelation efficacy of the anthracyclines is probably related to their marked lipophilicity (Miura et al., 1991), which enables rapid intracellular access in comparison to DFO which is hydrophilic and poorly penetrates cells (Richardson and Milnes, 1997).

In the current investigation, increased expression of *TfR1* and *Ndrg1* mRNA acted as a sensitive indice of intracellular Fe chelation and could be inhibited by pre-saturating the Fe-binding site of anthracyclines with Fe (Figure 4A). These Fe complexes still entered cells as they are highly hydrophobic (Miura et al., 1991) and this was obvious from the red color of the cell pellets which are usually white. Hence, the formation of the Fe complex prevented intracellular Fe chelation, but did not stop cellular access.

The nature of the Fe pools that regulate TfR1 and Ndrg1 expression remains unknown. However, these Fe pools influence IRP mRNA-binding activity which post-transcriptionally regulates *TfR1* mRNA (Hentze and Kuhn, 1996) and HIF-1α that transcriptionally up-regulates *TfR1*, *Ndrg1* and *VEGF1* (Beerepoot et al., 1996; Bianchi et al., 1999; Kalinowski and Richardson, 2005; Le and Richardson, 2004; Lok and Ponka, 1999). The DOX concentrations which up-regulate *TfR1* mRNA in SK-Mel-28 cells (*ie*., 1-2 μM; Figure 2A)

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were previously shown not to markedly affect IRP-mRNA-binding activity in this cell type (Kwok and Richardson, 2002), suggesting it was not an IRP response. Considering this, we also assessed the role of HIF-1α in regulating gene expression using HIF-1α knockout (*HIF-1α*$^{-/-}$) MEFs compared to their wild-type counterparts (*HIF-1α*$^{+/+}$). These studies suggested up-regulation of *TfR1*, *Ndrg1* and *VEGF1* mRNA by DOX occurred *via* an HIF-1α-independent mechanism, as regulation was comparable in the presence or absence of this transcription factor. Other studies examining HIF-1α activation by hypoxia also demonstrated that regulation of its target genes occurred irrespective of HIF-1α status in MEFs (Helton et al., 2005). Moreover, we showed using MEFs that DFO increased *Ndrg1* mRNA expression by HIF-1α-dependent and -independent mechanisms (Le and Richardson, 2004). Collectively, the current work and previous studies (Helton et al., 2005; Le and Richardson, 2004) indicated functional redundancy in the control of HIF-1α target gene expression, with a HIF-1α-independent mechanism responding to Fe chelation. This is of interest, as HIF-1α-independent pathways have been identified to be involved in the up-regulation of genes by hypoxia (Wood et al., 1998) and may also respond to Fe-depletion. Potentially, such pathways could be mediated by molecules related to HIF-1α, such as HIF-2α (Hu et al., 2003) and HIF-3α (Gu et al., 1998).

While anthracyclines could act like typical chelators such as DFO to bind Fe and induce up-regulation of Fe-responsive genes, the effect on cellular ^{59}Fe mobilization and intracellular ^{59}Fe distribution were atypical compared to other ligands. For instance, in contrast to DFO and PIH that induce cellular Fe efflux (Ponka et al., 1979; Richardson and Milnes, 1997), DOX had no effect on ^{59}Fe release from cells or cellular lysates at the same concentrations that up-regulated *TfR1* and *Ndrg1* mRNA. This suggests the high lipophilicity of DOX and its ^{59}Fe complex leads to marked retention in membranes and organelles, as shown by others (Hurwitz et al., 1997; Jung and Reszka, 2001; Miura et al., 1991).

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The multi-functional activity of DOX was shown by FPLC to lead to ferritin-^{59}Fe accumulation and prevent ^{59}Fe incorporation into high M_r compartments. This work confirmed and extended our previous observations demonstrating anthracyclines inhibit ferritin-Fe mobilization, which is probably mediated through inhibition of protein synthesis (Kwok and Richardson, 2003; Kwok and Richardson, 2004). Moreover, considering the alteration in ^{59}Fe distribution, it can be suggested that anthracycline-mediated Fe-deprivation which up-regulates *TfR1* and *Ndrg1* mRNA could not only be due to direct Fe chelation, but also to inhibition of ferritin-Fe mobilization.

An interesting observation which also demonstrated the multi-functional effect of DOX was that it acted as an effective protein synthesis inhibitor. This potentially could be responsible for the observed decrease in TfR1 and Ndrg1 protein as a function of DOX concentration. However, it was paradoxical that increasing DOX concentrations led to elevated ferritin protein expression, suggesting selective targeting of gene expression. This finding was surprizing, but was in accordance with previous studies demonstrating the effect of DOX at differentially targeting the expression of other genes (Chen et al., 1999; Ito et al., 1990). This selective activity of DOX has not been reported for genes involved or modulated by Fe metabolism. At present, it remains uncertain what precise molecular mechanism leads to DOX inhibiting TfR1 and Ndrg1 protein expression and increasing ferritin protein synthesis. The apparent selectivity in altering gene expression could be important for understanding the complex pharmacological effects of DOX.

As discussed above, the marked inhibition of TfR1 protein expression by DOX in SK-Mel-28 cells may be due to the depression of protein synthesis. Hence, this appeared to be a secondary response unrelated to Fe chelation which occurred after long preincubations with

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DOX that led to decreased Fe uptake from Tf. Certainly, the decreased TfR1 and increased ferritin protein expression observed after incubation with DOX is opposite to that found with typical Fe chelators such as DFO (Hentze and Kuhn, 1996) that are not potent protein synthesis inhibitors (Richardson and Milnes, 1997). Our current observations with neoplastic cells were in contrast to results using endothelial cells, where anthracyclines induced Fe uptake *via* increasing TfR1 protein (Kotamraju et al., 2002). These latter authors suggested that DOX-mediated apoptosis was accompanied by increased Fe uptake *via* TfR1 that was responsible for inducing apoptosis (Kotamraju et al., 2002). This result is controversial, as decreased intracellular Fe is generally associated with apoptosis and inhibiting proliferation (Kalinowski and Richardson, 2005).

In summary, anthracyclines act as atypical chelators up-regulating the mRNA expression of the Fe-regulated genes, *TfR1* and *Ndrg1* by their chelation of intracellular Fe. However, this complexation of Fe did not lead to increased TfR1 or Ndrg1 protein levels, nor did DOX induce cellular Fe mobilization. The lack of anthracycline-mediated Fe efflux was probably because of the high lipophilicity of the so-formed Fe complexes that remained intracellular. Considering the effect of anthracyclines on TfR1 and Ndrg1 expression, it was surprising and paradoxical that DOX increased ferritin protein expression and led to ferritin Fe accumulation. Hence, the effect of anthracyclines on Fe metabolism was multi-faceted, probably due to their complicated chemical properties which leads to multiple mechanisms of action.

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Footnotes

a) This project was supported by a fellowship and grants from the NHMRC of Australia.

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Figure Legends

Figure 1. (A) Schematic illustration of doxorubicin (DOX), epirubicin (EPI), daunorubicin (DAU) and the Fe complex of DOX. (B) Pre-incubation with DOX could not protect cells from the toxicity of subsequent Fe-loading by ferric ammonium citrate (FAC). Human SK-Mel-28 melanoma cells or rat H9c2 cardiomyocytes were pre-incubated with control medium (CON) or DOX (5 μM) for 24 h at 37°C and then washed. The cells were then reincubated for 16 h at 37°C with CON containing increasing concentrations of FAC (250, 500, 750 and 1000 μg/mL). Cell viability was examined using Trypan blue staining. The percentage of viable cells in control or DOX pre-treated group was plotted comparing to the relative control, which was set as 100%. Results are mean ± SD (3 experiments). *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ versus control values (Student's *t*-test).

Figure 2. DOX up-regulates *transferrin receptor-1* (*TfR1)* and *N-myc downstream regulated gene-1* (*Ndrg1*) mRNA levels in a concentration-dependent manner in a variety of tumor cell lines. (A) SK-Mel-28 melanoma cells, **(B)** SK-N-MC neuroepithelioma cells, **(C)** MCF-7 breast cancer cells, **(D)** DMS-53 lung carcinoma cells and **(E)** IMR-32 neuroblastoma cells were incubated with control medium (CON), DFO (100 μM) or DOX at increasing concentrations (0.5, 1, 2, and 5 μM) for 24 h at 37°C. The mRNA was then extracted and the expression of *TfR1* and *Ndrg1* mRNA levels were evaluated using RT-PCR. Densitometry was performed and gene expression was then calculated relative to the *β-actin* control. Results are a typical experiment from 3 experiments performed.

Figure 3. Anthracyclines increase *TfR1* and *Ndrg1* mRNA expression in a (A) dose-dependent and (B) time-dependent manner in SK-Mel-28 melanoma cells. (A) Cells were incubated for 24 h at 37°C with control medium (CON), ferric ammonium citrate (FAC; 100 μg/mL), desferrioxamine (DFO; 100 μM), doxorubicin (DOX; 2 μM), or daunorubicin (DAU)

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or epirubicin (EPI; 0.5, 1, 2 and 5 μM). The expression of *TfR1* and *Ndrg1* mRNA levels were evaluated using RT-PCR. **(B)** Cells were incubated with CON, DFO (100 μM), DOX (2 μM), DAU (2 μM) or EPI (2 μM) for 3, 6, 18 and 24 h. The expression of *TfR1* and *Ndrg1* mRNA levels were evaluated using RT-PCR. Gene expression was then calculated relative to the *β-actin* control. Results are typical from 3 separate experiments performed.

Figure 4. Anthracyclines up-regulate *TfR1* and *Ndrg1* mRNA levels by Fe-deprivation. (A) Anthracycline-Fe complexes are far less active than their parent ligands at increasing gene expression. (B, C) The soluble Fe salt, ferric ammonium citrate (FAC), decreases (B) *TfR1* and (C) *Ndrg1* mRNA expression after incubation with anthracyclines. (A) SK-Mel-28 cells were incubated for 24 h at 37°C with control medium (CON), DFO (2 μM), the 1:1 DFO-Fe complex (2 μM), DOX (2 μM), 3:1 DOX-Fe complex (2 μM), DAU (2 μM), 3:1 DAU-Fe complex (2 μM), EPI (2 μM) or 3:1 EPI-Fe complex (2 μM). **(B and C)** SK-Mel-28 cells were pre-incubated with CON, DFO (100 μM) or DOX (2 μM) for 20 h at 37°C (Primary Incubation), followed by a 20 h re-incubation at 37°C with CON, FAC (100 μg/mL), DFO (100 μM) or DOX (2 μM) (Secondary Incubation). The expression of *TfR1* and *Ndrg1* mRNA levels were evaluated using RT-PCR. Densitometric analysis was performed and gene expression was then calculated relative to the *β-actin* control. Results are typical of 3 experiments performed.

Figure 5. DOX does not act like a typical Fe chelator and cannot induce (A) ^{59}Fe efflux from intact cells or (B) effect ^{59}Fe mobilization from cellular lysates. However, DOX prevents ^{59}Fe mobilization from ferritin to other cellular compartments as shown by fast pressure liquid chromatography (FPLC) (C) and native PAGE ^{59}Fe-autoradiography (D). SK-Mel-28 cells were labeled for 3 h at 37°C with ^{59}Fe-Tf (0.75 μM), washed and then reincubated for 24 h at 37°C with control medium (CON), DFO (100 μM), PIH (25 μM) or

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DOX (0.1-5 μM). The overlying media and cells were collected and the ^{59}Fe levels examined. **(B)** SK-Mel-28 cells were labeled for 3 h at 37^{o}C with ^{59}Fe-Tf (0.75 μM) and the cells lysed, centrifuged and supernatant then isolated. The supernatant was then incubated with DFO (100 μM) or DOX (0.5-5 μM) for 3 h at 37°C and then subjected to ultrafiltration through a 5-kDa cut-off filter. The eluted fraction was collected and the radioactivity examined. **(C)** SK-Mel-28 cells were labeled for 24 h at 37^{o}C with ^{59}Fe-Tf (0.75 μM) in the presence or absence or DOX (2 μM) and the cellular lysates isolated as described in the *Materials and Methods*. The samples were then separated using a Superdex 200 10/300 GL size exclusion column. The radioactivity in each fraction (1 mL) was examined by a γ-counter. **(D)** Fraction 12 (F12) and 15 (F15) from **(C)** were assessed using 3-12% native-gradient-PAGE-^{59}Fe autoradiography. F15 contained ferritin which was confirmed by a super-shift experiment using an anti-ferritin antibody. Results in **(A)** and **(B)** are Mean ± SD (3 experiments), while data in **(C)** and **(D)** are a typical experiment from 3 performed.

Figure 6. (A) DOX mediated up-regulation of *TfR1* and *Ndrg1* mRNA occurs *via* a HIF-1α-independent mechanism. (B) DOX-generated reactive oxygen species are not involved in *TfR1* and *Ndrg1* mRNA up-regulation, but (C) plays a role in DOX-mediated protein synthesis inhibition. (A) Wild type (*HIF-1α*$^{+/+}$) and *HIF-1α*–knockout (KO; *HIF-1α*$^{-/-}$) murine embryo fibroblasts were incubated with control medium (CON), DFO (100 μM) or DOX (2 μM) for 8 h at 37^{o}C. The expression of *TfR1* and *Ndrg1* mRNA levels were then examined by RT-PCR. Densitometry was performed, and gene expression was then calculated relative to the *β-actin* control. **(B)** SK-Mel-28 cells were incubated with CON, DFO (100 μM) or DOX (2 μM) in the presence or absence of a combination of radical scavengers (RS) for 24 h at 37^{o}C. The RS included membrane impermeable agents, superoxide dismutase (SOD, 1000 U/mL) and catalase (1000 U/mL), the cell-permeable SOD mimetic MnTBAP (200 μM) and the cell-permeable glutathione peroxidase mimetic ebselen (15 μM). The expression of

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TfR1 and *Ndrg1* mRNA were examined using RT-PCR. Densitometry was performed as in **(A)**. **(C)** Cells were incubated with CON, DOX (5 μM), DAU (5 μM) or 5-imino-daunorubicin (5-i-D; 5 μM) for 22 h at 37°C and then ^{3}H-leucine (1 μCi/plate) was added into the media for 2 h at 37°C. The results in **(A)** and **(B)** are typical from 3 experiments, while the results in **(C)** are mean ± SD (3 experiments).

Figure 7. (A-C) DOX induces a dose-dependent reduction on both TfR1 and Ndrg1 protein levels, while ferritin-H and -L protein expression increases. (D) DOX increases *ferritin H*- and *L*-mRNA levels as a function of dose. (E) Pre-incubation with DOX results in decreased TfR1 protein expression that leads to depressed ^{59}Fe uptake from ^{59}Fe-transferrin, and (F) reduced incorporation of ^{59}Fe into ferritin protein. (A-C) SK-Mel-28 cells were incubated with control medium (CON), ferric ammonium citrate (FAC; 100 μg/mL), DFO (100 μM), or increasing concentrations of DOX (0.5-7.5 μM) for 24 h at 37°C. Western Blot was performed using anti-TfR1, anti-Ndrg1, anti-ferritin or anti-β-actin antibodies. **(D)** SK-Mel-28 cells were incubated with CON, DFO (100 μM) or DOX at increasing concentrations (0.5-5 μM) for 24 h at 37°C. The mRNA was then extracted and the expression of *ferritin-H and -L* mRNA levels were evaluated using RT-PCR. **(E)** SK-Mel-28 cells were pre-incubated with CON or DOX (2 μM) for 24 h at 37°C. This media was then removed and the cells then re-incubated for 0.5, 1, 2 and 4 h at 37°C with control media in the presence ^{59}Fe-Tf (0.75 μM). **(F)** Cell samples from **(E)** were lysed and native gradient PAGE-^{59}Fe-autoradiography then performed. The incorporation of ^{59}Fe into ferritin was confirmed by super-shift experiments using an anti-ferritin antibody. Results in **(A-D)** and **(F)** are typical from 3 experiments performed. The results in **(E)** are mean ± SD (3 experiments). $^{**}p < 0.01$ and *** $p < 0.001$ versus control values (Student's *t*-test).

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Table 1. Primers for amplification of human and mouse mRNA

Pair No.	Organisms	Target Gene	Accession No.	Oligonucleotides (5'-3')		Product Size (bp)
				Forward	Reverse	
1	*Homo sapiens*	*β-actin*	NM_001101	CCCGCCGCCAGCTCACCATGG	AAGGTCTCAAACATGATCTGGGTC	397
2		*Ndrg1*	NM_006096	CCCTCGCGTTAGGCAGGTGA	AGGGGTACATGTACCCTGCG	370
3		*TfR1*	NM_003234	GCTCGGCAAGTAGATGGC	TTGATGGTGCTGGTGAAG	359
4	*Mus musculus*	*β-actin*	NM_007393	CCCGCCACCAGTTCGCCATGG	AAGGTCTCAAACATGATCTGGGTC	397
5		*HIF-1α*	NM_010431	CTGGATGCCGGTGGTCTAGACAGT	CGAGAAGAAAAAGATGAGTTCTGAACGTCG	217
6		*Ndrg1*	NM_008681	TGCTTGCTCATTAGGTGTGTGATAGC	CCATCCTGAGATCTTAGAGGCAGC	581
7		*TfR1*	NM_011638	TCCCGAGGGTTATGTGGC	GGCGGAAACTGAGTATGATTGA	324
8		*VEGF1*	NM_009505	CCATGCCAAGTGGTCCCAG	GTCTTTCTTTGGTCTGCATTCACAT	346

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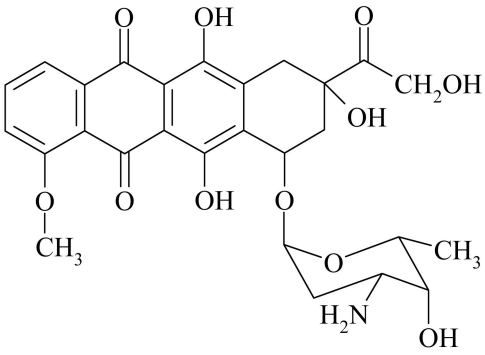
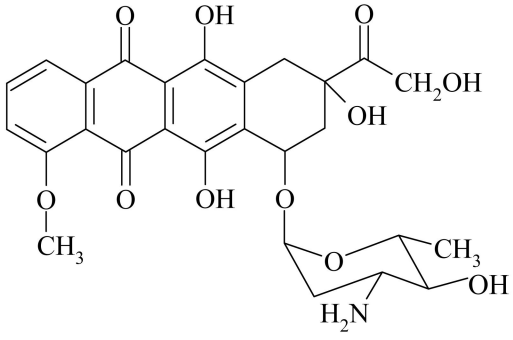
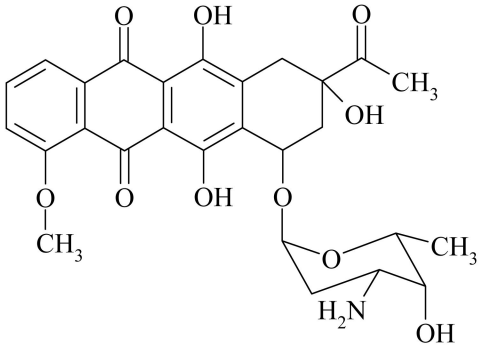
A)



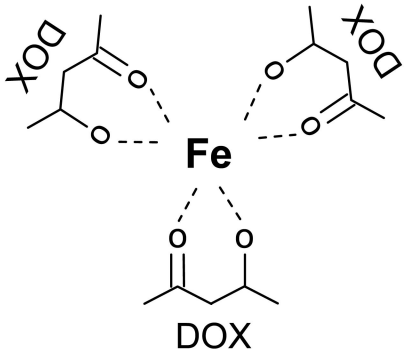
Doxorubicin (DOX)



Epirubicin (EPI)



Daunorubicin (DAU)



Fe complex of DOX

B)

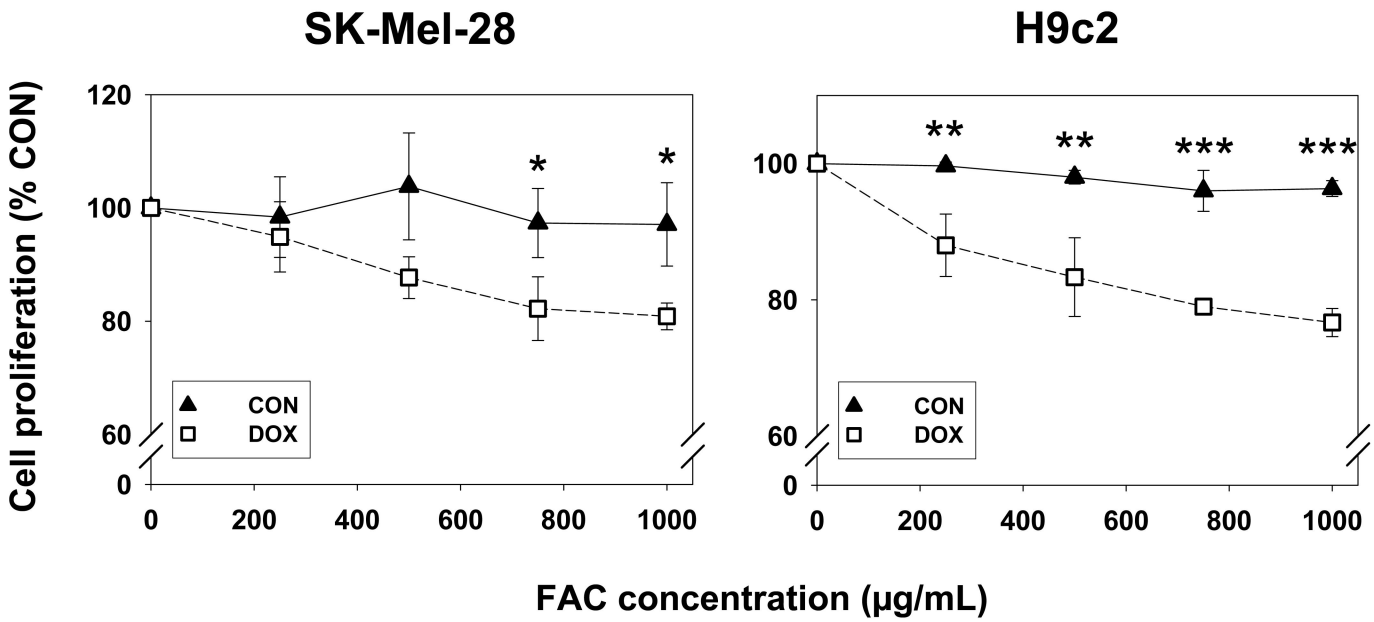


Figure 1

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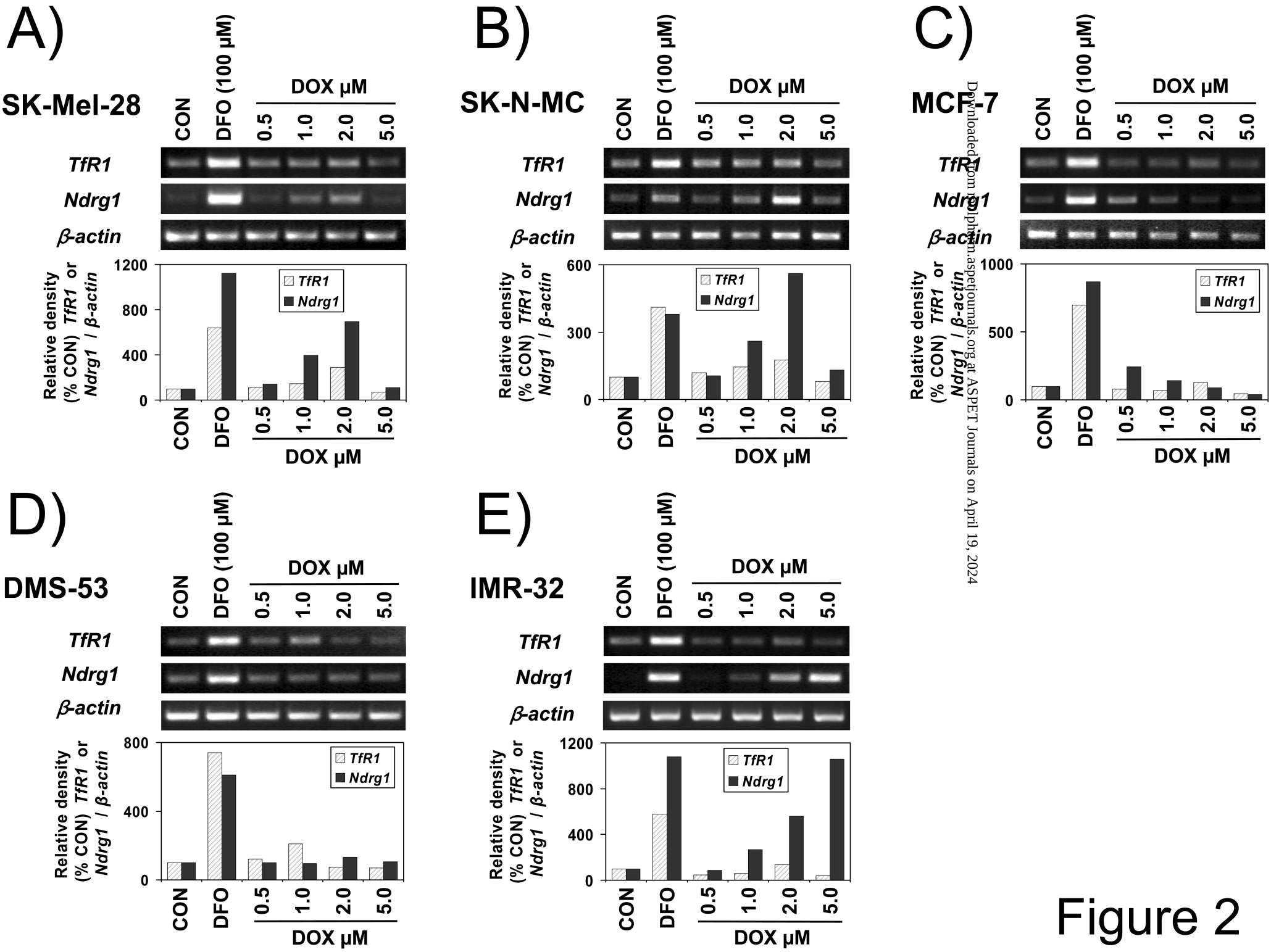

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Figure 2

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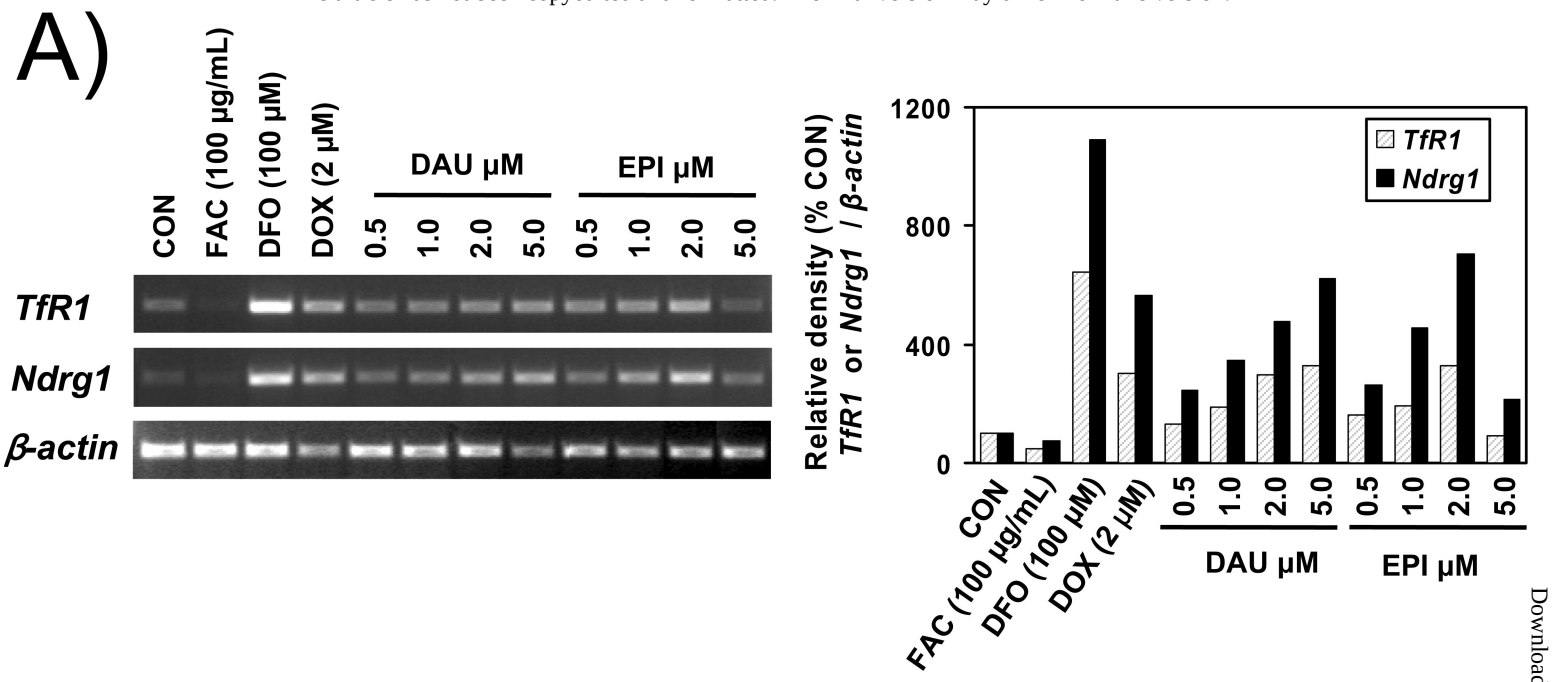

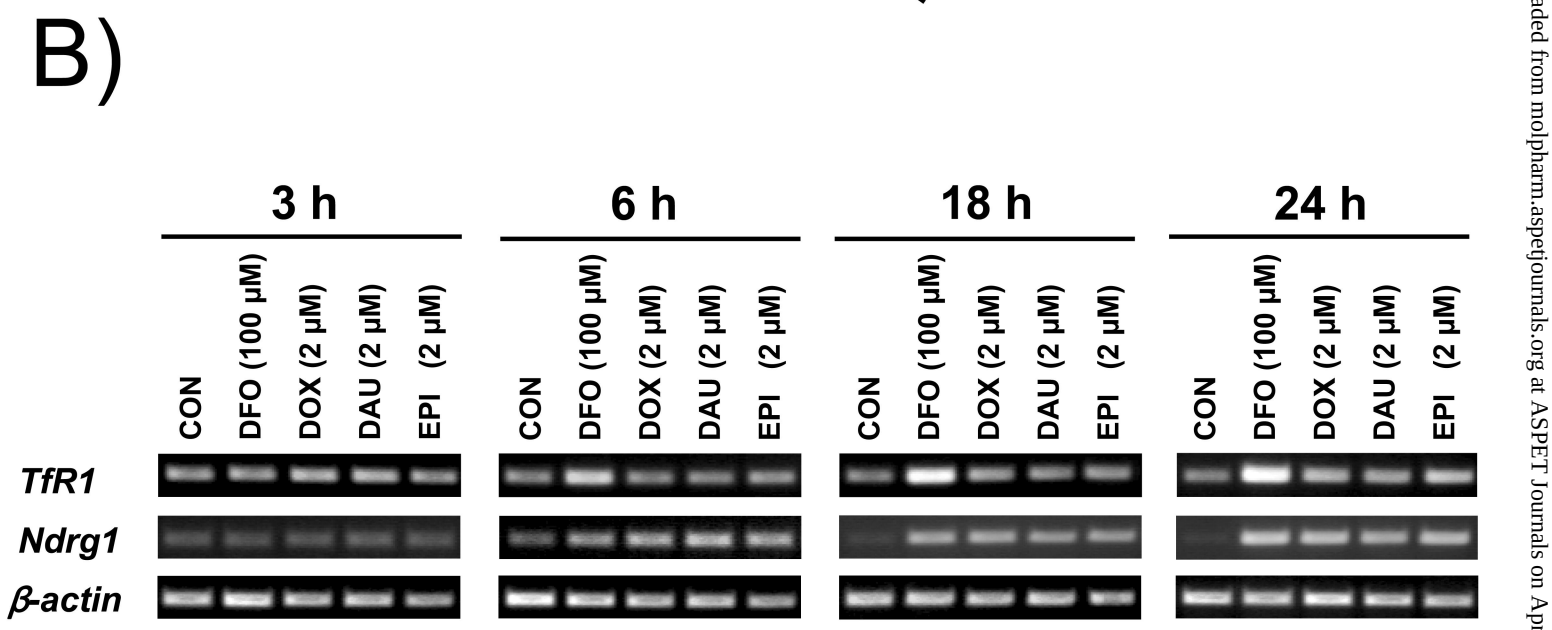

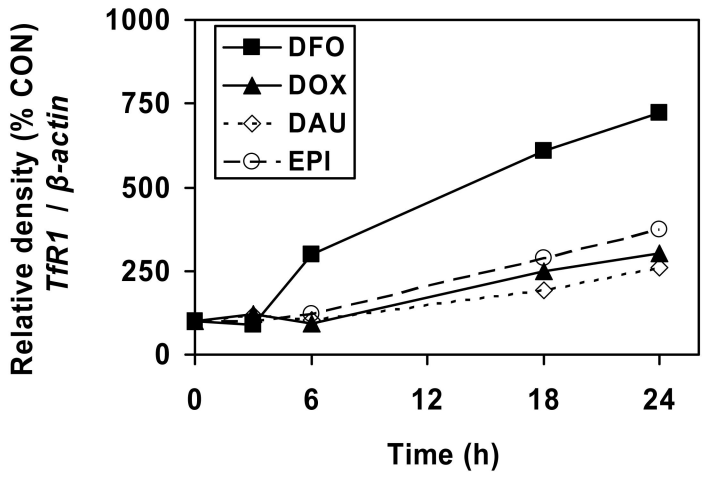

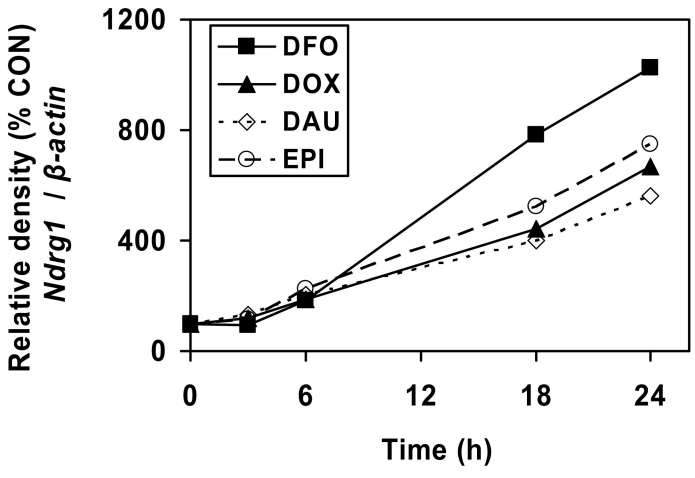

Figure 3

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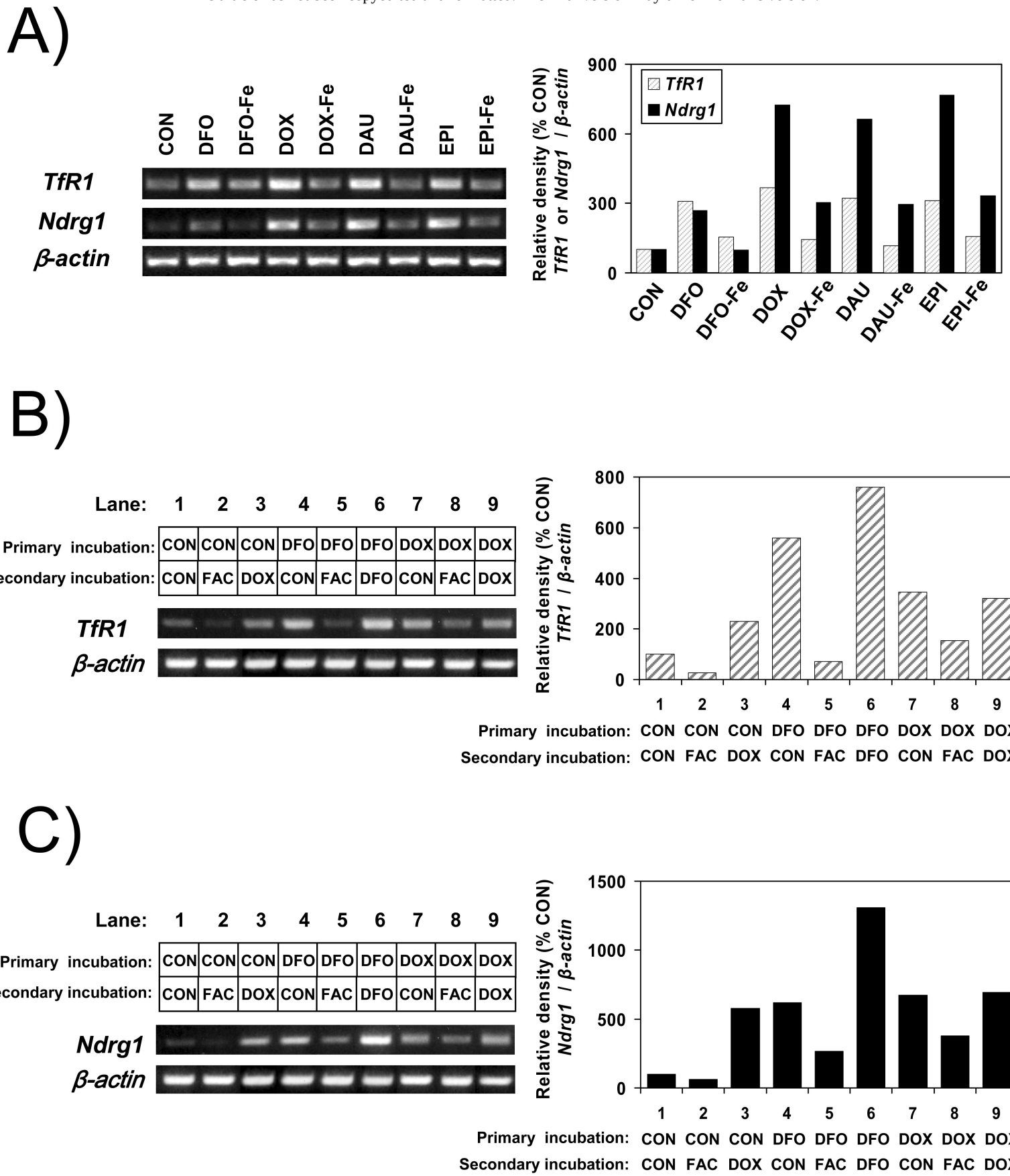

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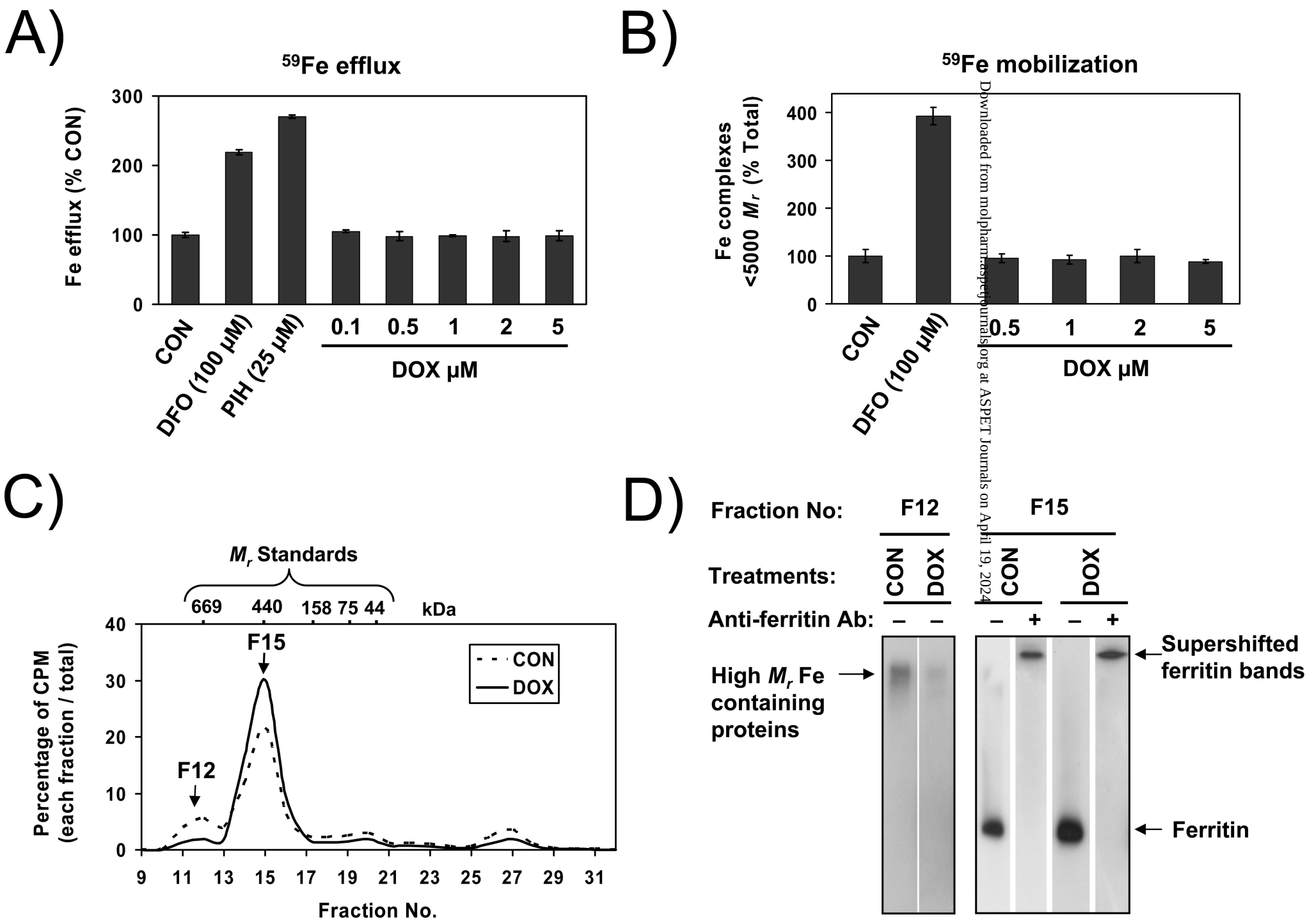
Figure 4



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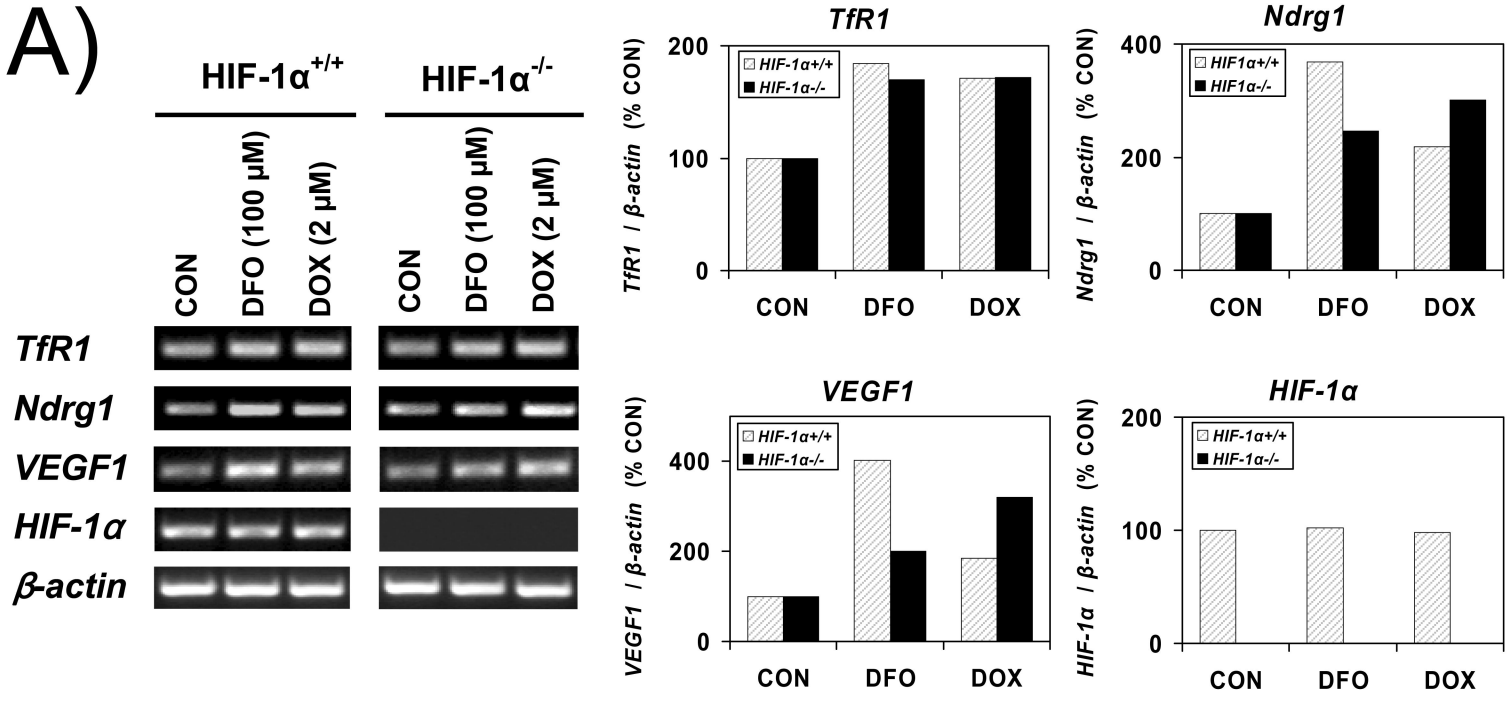

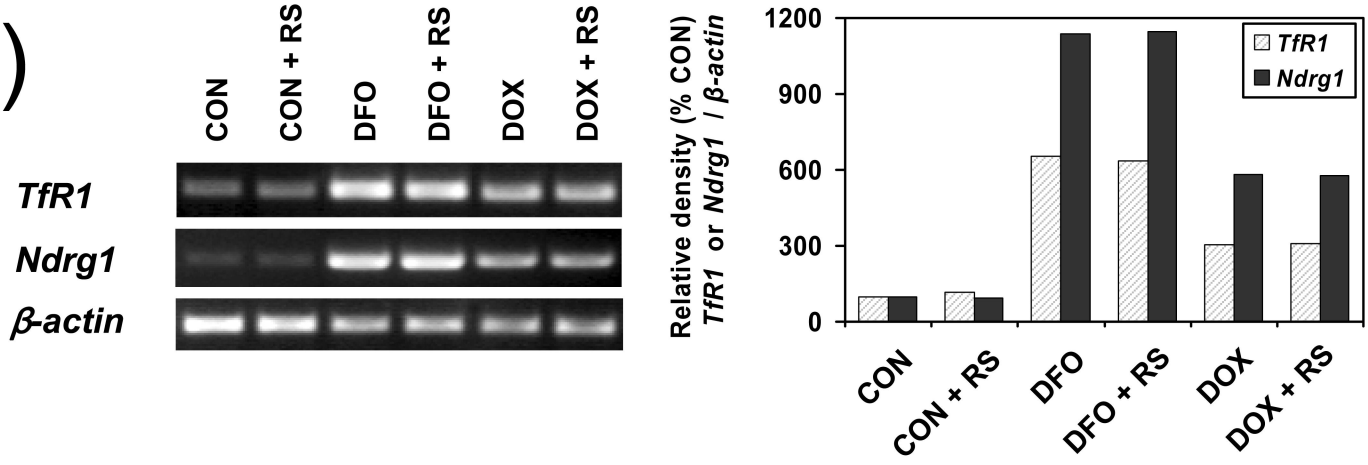

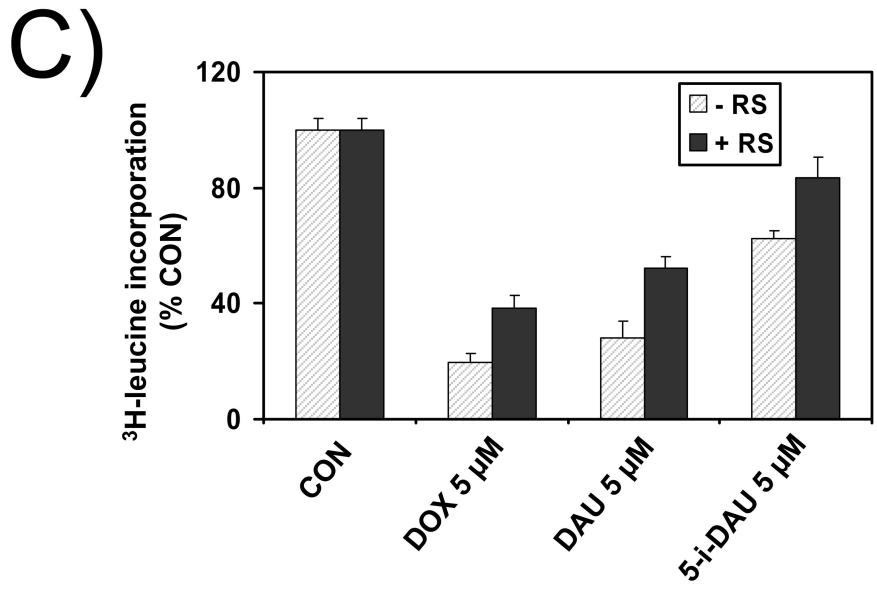

Radical Scavenger Combination (RS):

- catalase (1000 U/ml)
- ebselen (15 μM)
- MnTBAP (200 μM)
- SOD (1000 U/ml)

Figure 6

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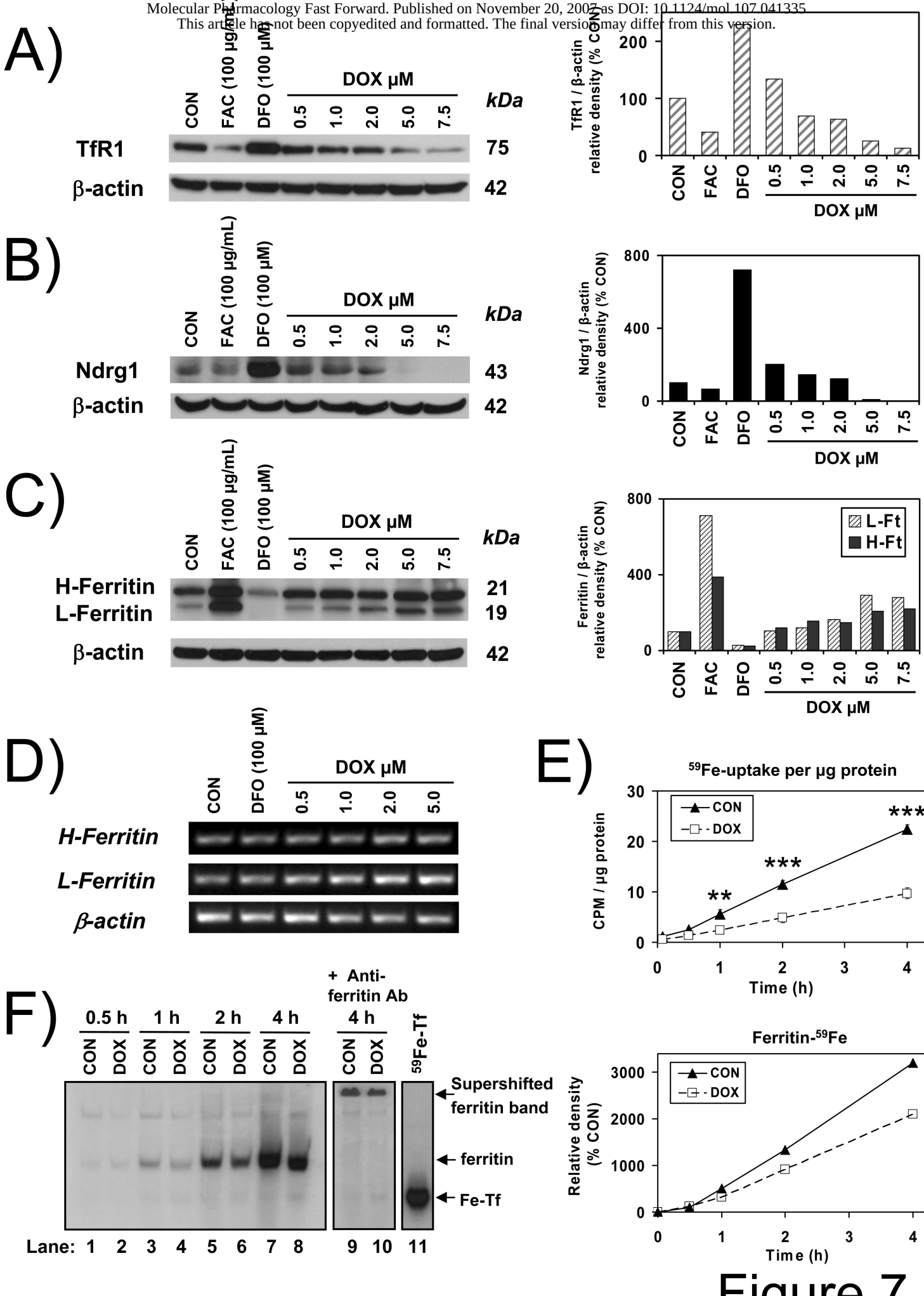

Figure 7

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