Figure 3.2 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^{o}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 3.5 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 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°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°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 3.6 (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 *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^{o}C and then ^{3}H-leucine (1 μCi/plate) was added into the media for 2 h at 37^{o}C. The results in **(A)** and **(B)** are typical from 3 experiments, while the results in **(C)** are mean $\pm$ SD (3 experiments).

Figure 3.7 (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^{o}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^{o}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^{o}C. This media was then removed and the cells then re-incubated for 0.5, 1, 2 and 4 h at 37^{o}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 $\pm$ SD (3 experiments). $^{**}p < 0.01$ and $^{***}p < 0.001$ versus control values (Student's *t*-test).