COUPLING OF THE POLYAMINE AND IRON METABOLISM PATHWAYS IN THE REGULATION OF PROLIFERATION: MECHANISTIC LINKS TO ALTERATIONS IN KEY POLYAMINE BIOSYNTHETIC AND CATABOLIC ENZYMES

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Abstract

Many biological processes result from the coupling of metabolic pathways. Considering this, proliferation depends on adequate iron and polyamines, and although iron-depletion impairs proliferation, the metabolic link between iron and polyamine metabolism has never been thoroughly investigated. This is important to decipher, as many disease states demonstrate co-dysregulation of iron and polyamine metabolism. Herein, for the first time, we demonstrate that cellular iron levels robustly regulate 13 polyamine pathway proteins. Seven of these were regulated in a conserved manner by iron-depletion across different cell-types, with four proteins being down-regulated (i.e., acireductone dioxygenase 1 [ADI1], methionine adenosyltransferase 2α [MAT2α], Antizyme and polyamine oxidase [PAOX]) and three proteins being up-regulated (i.e., S-adenosyl methionine decarboxylase [AMD1], Antizyme inhibitor 1 [AZIN1] and spermidine/spermine-N\(^1\)-acetyltransferase 1 [SAT1]). Depletion of iron also markedly decreased polyamine pools (i.e., spermidine and/or spermine, but not putrescine). Accordingly, iron-depletion also decreased S-adenosylmethionine that is essential for spermidine/spermine biosynthesis. Iron-depletion additionally reduced \(^3\)H-spermidine uptake in direct agreement with the lowered levels of the polyamine importer, SLC22A16. Regarding mechanism, the “reprogramming” of polyamine metabolism by iron-depletion is consistent with the down-regulation of ADI1 and MAT2α, and the up-regulation of SAT1. Moreover, changes in ADI1 (biosynthetic) and SAT1 (catabolic) partially depended on the iron-regulated changes in c-Myc and/or p53. The ability of iron chelators to inhibit proliferation was rescuable by putrescine and

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Conflict of interest

D.R.R. is a stakeholder in the companies, Oncochel Therapeutics LLC and Pty Ltd, which are developing the chelator, DpC, for the treatment of advanced and resistant solid tumors.
spermidine, and under some conditions by spermine. Collectively, iron and polyamine metabolism are intimately coupled, which has significant ramifications for understanding the integrated role of iron and polyamine metabolism in proliferation.

**Keywords**
Polyamines; acireductone dioxygenase 1 (ADI1); spermidine/spermine-\(N^1\)-acetyltransferase 1 (SAT1); iron; ornithine decarboxylase; S-adenosylmethionine (AdoMet).

1. **Introduction**

Polyamines are ubiquitous poly-cationic amines present in all cells and organisms [1–3]. The most common polyamines are putrescine, spermidine and spermine, which are major regulators of proliferation [4] (Fig. 1). Similarly to iron [5], polyamines are vital for proliferation and cell-cycle progression [6, 7] and many disease processes demonstrate co-dysregulation of iron and polyamine metabolism [1, 5–10].

While previous preliminary studies suggested a tentative link between iron chelation and polyamine metabolism, this relationship has never been thoroughly examined [11–13]. In fact, these studies only demonstrated altered activity and/or mRNA levels of three enzymes involved in polyamine metabolism: ornithine decarboxylase (ODC), spermidine/spermine-\(N^1\)-acetyltransferase 1 (SAT1) and methionine adenosyltransferase-2\(\alpha\) (MAT2\(\alpha\)), after iron chelation [11–14]. Importantly, examination of the relationship between iron and polyamine metabolism will provide insights into the mechanisms by which iron regulates proliferation. This is underscored by: (1) the ability of iron chelating agents to decrease cellular iron and abrogate proliferation [15]; and (2) the fact that chelators are being developed for cancer treatment [16].

The polyamine pathway can be divided into the “biosynthesis” (blue), “catabolism” (red) and “transport” (green) arms (Fig. 1). These arms can be sub-divided as follows: (1) the methionine salvage pathway; (2) ODC-Antizyme-Antizyme inhibitor 1 (AZIN1) axis; (3) biosynthetic core; (4) polyamine catabolism; and (5) polyamine transport (i.e., uptake and efflux) pathways. Classically, polyamine biosynthesis is focused on ODC, which is one of two rate-limiting enzymes in the polyamine pathway (Fig. 1). ODC catalyzes the decarboxylation of ornithine to putrescine [1, 3] (Fig. 1). The most abundant intracellular polyamines (i.e., spermidine and spermine) are biosynthesized from putrescine (Fig. 1).

In addition to the transcriptional activation of ODC by the oncogenic transcription factor, c-Myc [17–19], and the transcriptional repression by the tumor suppressor, p53, ODC is regulated at the level of protein degradation by a direct interaction with ODC Antizyme 1 (Antizyme) [1, 3]. When polyamine levels increase, the translation of Antizyme mRNA is enhanced, leading to a rapid increase in Antizyme protein [1, 3] (Fig. 1). Antizyme binds to the catalytically-active ODC dimer and promotes its dissociation into monomers, leading to inactivation of enzyme activity, which is followed by ODC degradation via the proteasome [20–22]. Apart from the translational regulation of Antizyme by polyamines, the ODC parologue, AZIN1, binds to Antizyme and prevents binding of Antizyme to ODC [20] (Fig.
1). Thus, AZIN1 positively regulates ODC protein levels by preventing Antizyme-dependent proteasomal degradation of ODC [20].

Spermidine and spermine are synthesized from the parental polyamine, putrescine, by the enzymes spermidine synthase (SRM) and spermine synthase (SMS) [1, 3]. A by-product of these reactions, 5′-methylthioadenosine, must be recycled back to methionine (Met) by the methionine salvage pathway [23]. Methionine is then condensed with ATP to form S-adenosylmethionine (AdoMet) by MAT2α, following which AdoMet is then decarboxylated to form decarboxylated AdoMet (dcAdoMet) by AdoMet decarboxylase (AMD1; Fig. 1). The iron-containing enzyme, acireductone dioxygenase 1 (ADI1; Fig. 1), is required for the recycling of methionine and the generation of dcAdoMet [23].

Polyamine levels are also regulated via their catabolism [1, 3]. Polyamine catabolism involves acetylation and oxidation of spermidine and spermine to putrescine and spermidine, respectively, or the direct oxidation of spermine to spermidine [24] (Fig. 1). The acetylation of spermidine and spermine by SAT1, yields N⁴-acetylspermidine and N⁴-acetylsperridine, respectively [25]. These acetylated polyamines can be oxidized by N¹-acetylpolyamine oxidase (PAOX), generating putrescine that can be released by the cell [24]. A third polyamine catabolic enzyme, spermine oxidase (SMOX), catalyzes the oxidation of spermine to spermidine [24] (Fig. 1).

Polyamines are also regulated at the level of transport. Export of spermidine and spermine is achieved through the efflux of their acetylated forms [26] (Fig. 1). These pools of acetylated polyamines are exported via the plasma membrane transporter, SLC3A2 [27] (Fig. 1), or other mechanisms [28]. On the other hand, polyamine uptake is mediated by the polyamine transport system [1, 3, 28]. A recently identified component of this system is the human solute carrier family 22, member 16 (SLC22A16; Fig. 1).

Similarly to polyamines, iron is essential for proliferation, including its crucial role in the rate-limiting step of DNA synthesis [29]. Iron-depletion is associated with a G₁/S arrest [29]. However, cellular iron status has been shown to affect a wide variety of molecular targets that play roles in cell cycle control, proliferation and metastasis suppression (e.g., cyclin D1, p21CIP1/WAF1, p53 and N-myc downstream regulated gene-1 [14, 30–35]). In fact, iron-depletion is potently tumor suppressive via multiple mechanisms, including up-regulation of p53, etc. [29–31, 33, 36–39].

The role of iron in proliferation has also been shown by the demonstration that specific, high affinity iron chelators, such as desferrioxamine (DFO) and 2-hydroxy-1-naphthylaldehyde isonicotinoyl hydrazone (311), can inhibit tumor cell proliferation by inducing cellular iron mobilization and inhibiting iron uptake from transferrin by cells [15, 16, 29, 40]. In fact, a chelator [i.e., di-2-pyridylketone 4-cyclohexyl-4-methyl-3-thiosemicarbazone (DpC)] has entered multi-center clinical trials for the treatment of advanced and resistant cancers [41, 42].

Herein, we identify multiple levels of regulation of polyamine metabolism by iron-depletion, involving polyamine biosynthesis, catabolism and transport. These changes suggest coordinated mechanisms by which iron-depletion leads to decreased spermidine and/or
spermine levels in cancer cells. We also identified key conserved changes that occur downstream of iron-dependent alterations in c-Myc and p53. Our novel findings are relevant to the major mechanisms by which iron-depletion exhibits potent anti-cancer activity [5, 43, 44].

2. Materials and methods

2.1 Cells

The following two human cell-types: melanoma, SK-Mel-28 and breast adenocarcinoma, MCF-7 cells were purchased from the American Type Culture Collection (ATCC; Manassas, VA). In some studies, experiments were validated using human hepatocellular carcinoma, HepG2 cells, human HaCaT keratinocytes, human breast cancer, MDA-MB-231, and human colon cancer, Caco-2 cells, all obtained from the ATCC. These cell-types were authenticated based on viability, recovery, growth, morphology and cytogenetic analysis by the provider.

2.2 Cell Treatments and Protein Extraction

SK-Mel-28 and MCF-7 cells were grown under standard conditions (37°C, 95% air/5% CO₂) in medium containing 10% (v/v) fetal calf serum (FCS; Life Technologies, Victoria, Australia) until 70% confluent. Subsequently, cells were treated for 24 h or 48 h at 37°C with control (media), 311 (25 µM), DFO (100 µM), or FAC (100 µg/mL). Proteins were then extracted and western blot analysis performed.

In some experiments, SignalSilence® c-Myc siRNA I (Cat. #: 6341; Cell Signaling Technology; Danvers, MA) and TP53 MISSION® endonuclease-digested siRNA pools (esiRNA; Cat. #: EHU123221; Sigma-Aldrich) were used, with comparisons made to a Silencer Negative control siRNA (“siNC”; Cat. #: AM4635; Life Technologies) or MISSION® FLUC esiRNA (“siNC*”; Cat. #: EHUFLUC; Sigma-Aldrich) respectively. The siRNAs were used at a final concentration of 2.5–20 nM, depending on the experiment. In all siRNA experiments, the siRNA-containing medium was removed and the cells were then incubated with control (media), DFO (100 µM), or ferric ammonium citrate (FAC; 100 µg/mL) for a further 48 h/37°C. Total proteins were then extracted and western blot analysis performed.

2.3 Western Blot Analysis

Proteins were separated on SDS-PAGE Tris-HCl 10% or 12%, 1.5 mm gels (Bio-Rad) and then transferred to PVDF membranes. Primary antibodies used in this study include: TfR1 (Cat. #: 136800; 1:2,000) from Life Technologies (Carlsbad, CA); AMD1 (Cat. #: PA5–31540; 1:1,000), from ThermoFisher Scientific (Waltham, MA); AZIN1 (Cat. #: ab57169; 1:500), ODC (Cat. #: ab97395; 1:1,000), Antizyme (Cat. #: ab85221; 1:100), MAT2α (Cat. #: ab77471; 1:2,000), ADI1 (Cat. #: ab37877; 1:1,000) and ADI1 (Cat. #: ab154689; 1:1,000) from Abcam (Cambridge, UK); PAOX (Cat. #: LS-B12847; 1:1,000) from LifeSpan Biosciences, Inc. (Seattle, WA); SAT1 (Cat. #: NB110–41622; 1:1,000) from Novus Biologicals (Littleton, CO); SMOX (Cat. #: 15052–1-AP; 1:1,000) from Proteintech (Rosemont, IL); c-Myc (Cat. #: 5605; 1:1,000), p53 (Cat. #: 48818; 1:1,000) from Cell Signaling Technology (Danvers, MA); SLC22A16 (Cat. #: HPA036902; 1:1,000), SLC3A2
(Cat. #: HPA017980; 1:1,000), SMS (Cat. #: HPA029849, 1:1,000), SRM (Cat. #: HPA029528; 1:1,000) and β-actin (Cat. #: A5316; 1:5,000) were from Sigma-Aldrich (St. Louis, MO). The secondary antibodies implemented include: horseradish peroxidase (HRP)-conjugated anti-rabbit (Cat. #: A6154; 1:3,000–1:10,000), anti-mouse (Cat. #: A4416; 1:5,000–10,000) and anti-chicken (Cat. #: A9046; 1:5,000) antibodies were from Sigma-Aldrich. The membranes were probed for β-actin to ensure equal protein loading. The protein bands were visualized using ECL Plus reagent (Pierce Chemical Co.). Images were captured using a Chemidoc System (Bio-Rad) and exposures were always made in the linear range to ensure quantitative estimation.

2.4 Determination of AdoMet (S-Adenosyl Methionine (SAM)) levels

The determination of AdoMet levels were assessed using the Bridge-It® SAM Fluorescence Assay (Mediomics LLC, St. Louis, MO), based on the manufacturer’s instructions.

2.5 RNA extraction and RT-PCR

TRIzol® (Invitrogen) reagent was used to isolate total RNA. For RT-PCR, 120 ng of total RNA was used to synthesize cDNA with ODC gene specific primers designed using Primer Premier (Premier Biosoft, Palo Alto, CA) at 0.4 µM final primer concentration. PCR cycles were performed on a MJ Research PTC-200 Peltier Thermal Cycler (Geneworks, NSW, Australia). Expression levels were normalized to β-actin mRNA which was used as a loading control.

2.6 Polyamine extraction, dansylation, separation and quantification by HPLC-MS/MS

The extraction and dansylation and subsequent HPLC-MS/MS analysis of putrescine, spermidine and spermine was performed by the method of [45]. Polyamine levels were calculated as nmol/mg protein as % control.

2.7 3H-spermidine uptake studies

SK-Mel-28 and MCF-7 cells were seeded onto 6 well plates and grown for 48 h/37°C. Both cell-types were treated with either media (Control) containing 10% (v/v) FCS (Life Technologies), or this media containing 311 (25 µM), DFO (100 µM), FAC (100 µg/mL) with/without DFMO (5 mM) for a further 24 h/37°C. After treatment, the cells were washed twice with serum-free media containing 1 mg/mL BSA. This was followed by incubating the cells with 3H-spermidine (1 µM; specific activity: 23.6 Ci/mmol and 873.2 GBq/mmol; Cat. #: NET522001MC; Perkin Elmer) for 1 h/37°C. The cells were then washed twice with ice-cold PBS and incubated in Pronase (1 mg/mL; Sigma-Aldrich) for 30 min/4°C to separate membrane associated radioactivity from internalized radioactivity [46–48].

2.8 Proliferation/viability assays

Proliferation was examined using the [3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2H-tetrazolium] (MTT) assay [49]. The optical absorbance resulting from the presence of the insoluble purple formazan was directly proportional to the number of viable cells, as validated by viable cell counts using the Trypan blue exclusion assay [15].
2.9 Statistics

Densitometry of western blot results were expressed as mean ± standard deviation (SD). All experiments were conducted at least 3 times and compared using 1-way ANOVA with Fisher’s least significant difference post-hoc test. Results were considered statistically significant when \( p < 0.05 \).

3 Results

3.1 Iron-depletion robustly alters polyamine metabolism at multiple levels

Examining MCF-7 cells, our laboratory reported [11–14] that two key polyamine pathway genes (i.e., MAT2A and ODC1) were down-regulated at the mRNA level following iron-depletion with the iron chelators, 311 or DFO [15, 40, 50–52]. Thus, we hypothesized that iron-depletion may regulate other key proteins involved in the polyamine pathway (Fig. 1).

In general, polyamine metabolism and cellular polyamine content are tightly controlled by the change in abundance of key enzymes and proteins [1]. Therefore, to assess whether modulation of cellular iron levels affected polyamine metabolism, we first assessed the expression of all major polyamine pathway proteins (Figs. 2–6). To determine the effects of modulating iron levels on the expression of polyamine pathway proteins, we employed SK-Mel-28 and MCF-7 cells, as their iron metabolism is well characterized and the effects of chelators on these cell-types have been thoroughly examined [46, 47, 52–55]. Cells were incubated with control medium, or this medium containing the well-characterized iron chelators, 311 (25 μM), or DFO (100 μM) [15, 16, 29, 40], or the cellular iron donor, FAC (100 μg/mL) [46, 56, 57], for 24 or 48 h/37°C (Figs. 2–6). These incubations were implemented to assess “early” (by 24 h) and “late” (by 48 h) responses of the polyamine pathway to iron-depletion/supplementation. After these incubations, the cell monolayer remained viable and intact.

Both DFO and 311 deplete intracellular iron [15, 52] that markedly increases iron regulatory protein (IRP) RNA-binding activity, which induces up-regulation of the transferrin receptor 1 (TfR1) [50]. In contrast, incubation with FAC is known to donate iron to intracellular iron pools, leading to decreased IRP-RNA-binding activity that down-regulates TfR1 [46, 58–60]. As such, in all studies, the functional regulation of the iron-sensitive positive control, TfR1, by cellular iron levels [61, 62], was monitored. Western analysis was then used to assess the relative abundance of the major polyamine pathway enzymes/proteins (Figs. 2–6).

Using both cell-types, incubation with 311 or DFO, led to a marked and significant (\( p < 0.001 \)) increase in TfR1, demonstrating iron-depletion (Figs. 2–6). In contrast, incubation with FAC led to a significant (\( p < 0.001 – 0.05 \)) decrease in TfR1 (Figs. 2–6), indicating repletion of iron pools. Herein, we demonstrate that iron-depletion regulated 13 major polyamine metabolism proteins (Figs. 2–6). As described below, the changes in expression of polyamine pathway proteins were accompanied by corresponding decreases in specific and overall polyamine levels (described later in Fig. 7A, B). Of the 13 polyamine pathway proteins regulated by iron-depletion (Figs. 2–6), seven were regulated similarly by iron-depletion in both SK-Mel-28 and MCF-7 cells (conserved changes). In fact, four proteins were significantly (\( p < 0.001 – 0.01 \)) down-regulated (i.e., ADI1, MAT2α, Antizyme and
PAOX) and three significantly \((p < 0.001 – 0.05)\) up-regulated \((i.e., \text{AMD1, AZIN1 and SAT1})\) in both cell-types by iron-depletion (Figs. 2, 3, 5). Notably, as many key polyamine pathway proteins are regulated by polyamine levels [1], the changes described in Figs. 2–6 include: \((i)\) possible effectors of, and \((ii)\) predicted responses to, the decrease in polyamines caused by iron-depletion described in Fig. 7A, B.

### 3.2 Methionine salvage pathway (AD11, MAT2α, and AMD1)

Incubation of both cell-types with 311 or DFO for 24 or 48 h led to a significant \((p < 0.001 – 0.01)\) decrease in AD11 and MAT2α. (Fig. 2A, B). The decreased expression of AD11 and MAT2α was time-dependent, being more pronounced at 48 h than 24 h (Fig. 2A, B). While AD11 is crucial for methionine regeneration in the methionine salvage pathway [23], MAT2α is required for the ATP-dependent conversion of methionine into AdoMet (Fig. 1) [63]. In fact, AdoMet is a substrate for the next enzyme in the biosynthetic sequence, AMD1 (Fig. 1), which biosynthesizes dcAdoMet that is vital for spermidine/spermine biosynthesis [63].

Due to the decrease in MAT2α expression in response to iron-depletion (Fig. 2A, B), AdoMet production would be predicted to be impaired. As such, incubation of SK-Mel-28 cells with DFO for 24 h led to a reduction in AdoMet levels \((i.e., \text{control: } 3.5 \pm 0.1 \text{ (3) pmol/mg protein vs DFO: } 0.3 \pm 0.8 \text{ (3) pmol/mg protein})\). A similar, although less marked reduction in AdoMet levels was also observed for MCF-7 cells incubated with DFO for 24 h \((i.e., \text{control: } 3.0 \pm 0.4 \text{ (3) pmol/mg protein vs. DFO: } 1.2 \pm 0.7 \text{ (3) pmol/mg protein})\).

Decarboxylation of AdoMet by AMD1 generates dcAdoMet [63] (Fig. 1). This metabolite is required for spermidine and spermine synthesis [63]. Importantly, AMD1 is a key rate-limiting enzyme in spermidine/spermine biosynthesis (Fig. 1), and, like ODC, its levels are low, and are inducible by decreased polyamine levels [63]. After a 24 h incubation of SK-Mel-28 or MCF-7 cells with DFO or 311, only DFO significantly \((p < 0.01)\) increased AMD1 expression in SK-Mel-28 cells (Fig. 2A, B). In contrast, after a 48 h incubation, both chelators significantly \((p < 0.001 – 0.01)\) increased AMD1 expression in both cell-types (Fig. 2A, B). As AMD1 is translationally up-regulated by decreased polyamine levels [64], this result suggests an iron-depletion-dependent increase in AMD1 occurred as a homeostatic response to polyamine depletion.

We next considered the other biosynthetic branch of the polyamine pathway that is responsible for the generation of putrescine, namely: the ODC-Antizyme-AZIN1 axis [1] (Fig. 1).

### 3.3 The ODC-Antizyme-AZIN1 axis

This is the other rate-limiting step in polyamine biosynthesis, which consists of ODC, Antizyme and AZIN1 and controls putrescine levels (Fig. 1) [20, 65]. When considering the effects of iron levels on ODC expression, we observed differential regulation in SK-Mel-28 and MCF-7 cells following incubation with 311 or DFO for 24 or 48 h (Fig. 3A, B).

Typically, two closely migrating ODC bands were evident at ~45–50 kDa \((e.g., \text{Fig. 3A, B})\), which depended, in part, on the SDS-PAGE gel acrylamide percentage employed \((i.e., 10– \)
12% w/v). The slower-migrating upper band may be a post-translational modification of ODC (e.g., a phosphorylated form). In fact, physiologically-relevant phosphorylated forms of human ODC (e.g., at Ser-167) have been identified [66]. In the case of SK-Mel-28 cells, there was a significant (p < 0.001 – 0.01) increase in ODC at 24 and 48 h in response to 311 or DFO (Fig. 3A). In contrast, for MCF-7 cells, there was no significant (p > 0.05) change in ODC expression following incubation with 311 or DFO after 24 or 48 h, suggesting a cell-type dependent difference in the regulation of this axis (Fig. 3B). Notably, the major mechanism of ODC regulation in response to changes in polyamine levels is a change in the rate of its protein degradation [20]. As alterations in ODC degradation are primarily caused by changes in Antizyme and/or AZIN1 levels under other conditions [20] (Fig. 1), we next examined the expression of these key ODC regulators.

Antizyme expression is primarily down-regulated by a translational mechanism in response to a decrease in cellular polyamines [20]. As such, Antizyme levels were significantly (p < 0.001) decreased following iron-depletion of SK-Mel-28 and MCF-7 cells after 24 h and 48 h incubations with 311 or DFO (Fig. 3A, B). As with AMD1 up-regulation after iron-depletion (Fig. 2A, B), this pronounced decrease of Antizyme in both cell-types is consistent with a homeostatic response to a reduction in cellular polyamine levels [20]. These data suggest the increase in ODC in iron-depleted SK-Mel-28 cells may be due to the marked decrease in Antizyme levels (Fig. 3A). Despite the marked decrease in Antizyme, ODC levels were not significantly (p > 0.05) increased in iron-depleted MCF-7 cells (Fig. 3B), again indicating a different mechanism of regulation of ODC between the cell-types.

We next examined AZIN1 expression, which is a key positive regulator of ODC [20] (Fig. 1). AZIN1 acts to increase ODC expression and activity by binding to, and sequestering Antizyme [20]. Strikingly, AZIN1 was significantly (p < 0.001 – 0.05) up-regulated by 311 and DFO in both cell-types by 48 h (Fig. 3A, B). In SK-Mel-28 cells, AZIN1 was significantly and similarly up-regulated at 24 h (p < 0.001 – 0.01) and 48 h (p < 0.001) by 311 or DFO (Fig. 3A). On the other hand, for MCF-7 cells, the up-regulation of AZIN1 by chelators was more pronounced at 48 h (p < 0.001 – 0.05), relative to 24 h (p < 0.05, 311 only; Fig. 3B). Like AMD1, AZIN1 is known to be homeostatically up-regulated by low polyamine levels [20].

Given the differences in regulation of ODC protein by iron-depletion between the two cell-types, we also examined ODC mRNA levels (Suppl. Fig. 1A, B). There was a significant (p < 0.001 – 0.05) decrease in ODC mRNA following iron-depletion with 311 or DFO in both cell-types (Suppl. Fig. 1A, B). Thus, despite this down-regulation of ODC mRNA, ODC protein levels were either increased (Fig. 3A), or were unchanged (Fig. 3B). Collectively, there is probably an interplay in the regulation of ODC protein expression in each cell-type via alterations in its mRNA expression (Suppl. Fig. 1A, B), but also by the modulation in AZIN1 and Antizyme protein levels (Fig. 3A, B).

Overall, the changes in Antizyme and AZIN1 protein expression in both cell-types in response to DFO and 311 indicate a conserved response to decreased polyamines [20]. Regarding the differential effects of iron-depletion on ODC protein levels, it can be speculated that the more pronounced down-regulation of Antizyme in SK-Mel-28 cells (Fig.
3A), relative to MCF-7 cells (Fig. 3B), may have resulted in a net increase in ODC protein levels in the former cell-type. This is suggested as: (1) changes in ODC protein degradation are mainly induced by alterations in Antizyme protein expression [20]; and (2) down-regulation of ODC mRNA levels upon incubation with DFO or 311 was similar in both SK-Mel-28 and MCF-7 cells (Suppl. Fig. 1A, B).

### 3.4 The biosynthetic core (SRM and SMS)

SRM and SMS are sequential enzymes catalyzing the production of spermidine and spermine from putrescine and spermidine, respectively (Fig. 1; [1]). The aminopropyl donor required for these reactions is dcAdoMet, which is generated by AMD1 (Fig. 1). As spermidine and spermine are the major polyamines [1], these reactions form the “biosynthetic core” of polyamine metabolism.

When considering the effects of iron levels on these enzymes, we observed differential regulation assessing SK-Mel-28 and MCF-7 cells (Fig. 4A, B). Examining SK-Mel-28 cells, iron-depletion by 311 or DFO led to a significant ($p < 0.001$) down-regulation of SRM at 24 and 48 h (Fig. 4A). In contrast, iron supplementation with FAC led to a significant ($p < 0.001$) up-regulation of SRM by 48 h in SK-Mel-28 cells. In the case of SMS, iron-depletion using 311 or DFO led to a significant ($p < 0.001 – 0.05$) and time-dependent, up-regulation of SMS in SK-Mel-28 cells (Fig. 4A).

In contrast, assessing MCF-7 cells, iron-depletion with 311 or DFO or iron supplementation by FAC had no significant effect on SRM levels (Fig. 4B). Considering SMS, by 48 h there was a slight, but significant ($p < 0.05$), down-regulation following iron-depletion with 311, but not DFO (Fig. 4B). No significant ($p > 0.05$) alterations in SMS were observed after incubation of MCF-7 cells with FAC.

### 3.5 Polyamine catabolism (SAT1, PAOX and SMOX)

We next considered the effects of iron levels on the polyamine catabolic enzymes, SAT1, PAOX and SMOX [24] (Fig. 1). This was important as polyamine catabolism is a major regulator of polyamine levels [24].

A highly inducible enzyme involved in polyamine catabolism is SAT1 (Fig. 1) [25]. This enzyme acetylates spermidine and spermine at the $N^1$ position, and is a major regulator of polyamine levels [25]. When considering the effect of iron levels on SAT1 expression, we observed a similar effect in both cell-types (Fig. 5A, B). In fact, there was a significant ($p < 0.001 – 0.01$) up-regulation of SAT1 relative to the control, by 48 h (SK-Mel-28 cells) (Fig. 5A), or by 24 and 48 h (MCF-7 cells; Fig. 5B), following incubation with 311 or DFO.

Importantly, the acetylated polyamine products of the SAT1-catalyzed reaction are either oxidized by PAOX, or excreted from the cell (e.g., by SLC3A2; Fig. 1). When PAOX levels were considered, significant ($p < 0.001$) down-regulation was observed in both SK-Mel-28 and MCF-7 cells after a 48 h incubation with 311 or DFO (Fig. 5A, B).

Like SAT1, SMOX is another inducible, and highly regulated, polyamine catabolic enzyme [24, 67] (Fig. 1). This oxidase acts to oxidize spermine to spermidine [67]. In SK-Mel-28
cells, SMOX levels were significantly \((p < 0.001)\) up-regulated at 24 and 48 h following incubation of cells with 311 or DFO, while they were significantly \((p < 0.01)\) down-regulated by FAC by 48 h (Fig. 5A). In contrast, in MCF-7 cells, SMOX expression was significantly \((p < 0.001–0.01)\) decreased by 48 h following incubation with 311 or DFO (Fig. 5B). Moreover, incubation of these cells with FAC led to significant \((p < 0.01)\) increase in SMOX by 48 h (Fig. 5B).

Overall, for both cell-types, SAT1 expression was increased by iron-depletion, which is consistent with increased \(N^1\)-acetylspermine or \(N^1\)-acetylspermidine biosynthesis. On the other hand, PAOX levels were generally decreased in SK-Mel-28 and MCF-7 cells, suggesting reduced conversion of these \(N^1\)-acetylated polyamines back to spermine and putrescine, respectively.

3.6 Polyamine transport (SLC22A16 and SLC3A2)

Intracellular polyamine levels are also regulated at the level of cellular uptake and efflux [28, 68]. Although multiple polyamine uptake mechanisms have been proposed [28, 68], one recently discovered pathway, involves the plasma membrane polyamine importer, SLC22A16 [69]. This protein may be responsible for the uptake of a substantial fraction of spermidine [69]. When the effect of iron levels on SLC22A16 expression in SK-Mel-28 cells was examined, a significant \((p < 0.001)\) down-regulation was observed at 24 and 48 h (Fig. 6A). In contrast, for MCF-7 cells, iron-depletion had no significant \((p > 0.05)\) effect on SLC22A16 levels (Fig. 6B). This observation suggests that iron-depletion may have suppressed the capacity for polyamine import in SK-Mel-28 cells, but not MCF-7 cells. Indeed, as described subsequently in reference to spermidine uptake in Fig. 7C, iron-depletion specifically impaired its import in SK-Mel-28 cells, but not MCF-7 cells.

We next examined the effect of iron on the levels of SLC3A2, which is an \(N^1\)-acetylspermidine exporter that forms a plasma membrane-associated metabolon with SAT1 [27]. In SK-Mel-28 cells, SLC3A2 levels were significantly \((p < 0.001)\) decreased following a 48 h incubation of cells with 311 or DFO (Fig. 6A). Notably, although there was no significant change in SLC3A2 levels after a 24 h incubation of SK-Mel-28 cells with 311, there was a significant \((p < 0.001)\) up-regulation of this protein following a 24 h incubation with DFO (Fig. 6A). In contrast, when assessing MCF-7 cells, iron-depletion with 311 or DFO led to a marked and significant \((p < 0.001)\) up-regulation of SLC3A2 by 48 h, while only 311 significantly \((p < 0.001)\) up-regulated SLC3A2 after 24 h (Fig. 6B).

Overall, in SK-Mel-28 cells, iron-depletion decreased the expression of proteins involved in polyamine uptake and efflux, suggesting polyamine transport was down-regulated by cellular iron levels (also see Fig. 7C). In contrast, using MCF-7 cells, iron-depletion had no effect on SLC22A16 expression (Fig. 6B), which is involved in polyamine uptake. However, the depletion of iron in MCF-7 cells increased levels of the polyamine exporter, SLC3A2 (Fig. 6B). This occurred together with the up-regulation of its metabolic-binding partner, SAT1 (Fig. 5B). On the other hand, the expression of SAT1 and SLC3A2 were not concordant in SK-Mel-28 cells, suggesting a different effect on this metabolon. Collectively, all conserved changes between the cell-types (Figs. 2–6) are consistent with the depression of polyamine levels after iron-depletion (described below).
3.7 Effect of iron-depletion on cellular polyamine levels

Given that iron-depletion markedly altered the expression of multiple polyamine pathway proteins (Figs. 2–6), we next assessed the effects of modulating iron on key polyamine concentrations. This was achieved by incubating SK-Mel-28 or MCF-7 cells with control medium, 311 (25 μM), DFO (100 μM), or the Fe donor, FAC, for 24 h or 48 h/37°C. Putrescine, spermidine and spermine, were then quantitatively assessed by derivatization, HPLC-mediated separation, and tandem mass spectrometry (Fig. 7A, B).

The absolute polyamine levels in SK-Mel-28 and MCF-7 cells incubated under control conditions for 24 h were (n = 3): SK-Mel-28: putrescine (0.4 ± 0.2 nmol/mg protein), spermidine (18.3 ± 0.4 nmol/mg protein) and spermine (89.8 ± 0.6 nmol/mg protein); and for MCF-7 cells: putrescine (0.3 ± 0.1 nmol/mg protein), spermidine (38.5 ± 11.2 nmol/mg protein) and spermine (307.9 ± 6.2 nmol/mg protein). Such polyamine levels are consistent with previous results in SK-Mel-28 cells [70] and MCF-7 cells [71]. Although previous investigations have demonstrated that spermine levels are similar to spermidine levels in MCF-7 cells, these prior studies used different growth conditions to the present work, namely: (i) cells were grown in 5% (v/v) FCS [71], relative to 10% (v/v) FCS in our studies; and (ii) cells were grown for >144 h [71], relative to 72 h in this investigation.

The effect of modulating iron on these polyamine profiles was then assessed. For SK-Mel-28 cells, 311, or DFO did not significantly (p > 0.05) alter putrescine or spermidine levels after 24 h (Fig. 7A). However, in the case of spermine, the incubation of SK-Mel-28 cells with 311 or DFO for 24 h significantly (p < 0.01) decreased this polyamine to 40–50% of control levels (Fig. 7A). On the other hand, incubation of cells with FAC resulted in no significant alteration in all polyamines. This is probably because the control cells were Fe-replete and adding extra iron as FAC results in no effect.

These alterations in polyamine levels in SK-Mel-28 cells after iron-depletion are consistent with the changes in expression of polyamine metabolic pathway proteins shown in Figs. 2A–6A. That is, the pronounced decrease in spermine after 24 h incubation with 311 or DFO (Fig. 7A) is consistent with the decline in ADI1 and MAT2α (Fig. 2A), which would limit supply of dcAdoMet for spermine biosynthesis (see Fig. 1). The decline in spermine in SK-Mel-28 cells is further predicted by the marked increase in SMOX (Fig. 5A), which acts to degrade spermine to spermidine.

As with the regulation of polyamine pathway proteins shown in Figs. 2B–6B, an overlapping, but distinct set of alterations in polyamine levels were observed in MCF-7 cells (Fig. 7B). Indeed, incubation of MCF-7 cells with 311 or DFO did not significantly (p > 0.05) alter putrescine after 24 h (Fig. 7B). However, in the case of spermidine and spermine, incubation of MCF-7 cells with 311 or DFO for 24 h significantly (p < 0.01) decreased their levels (Fig. 7B). In contrast, FAC resulted in a significant (p < 0.05) ~50% elevation in putrescine, while no alteration in other polyamines was observed. This increase in putrescine with iron supplementation is not explained by changes in ODC (Fig. 3B) or SRM expression (Fig. 4B), suggesting some other metabolic process that modulates putrescine levels may be affected by iron. Overall, the data in Figs. 2–7 indicate that polyamine metabolism is regulated by cellular iron-depletion.
3.8 Effect of iron-depletion on cellular $^3$H-spermidine uptake

The uptake of extracellular polyamines is also a major contributor to polyamine levels, particularly when polyamine biosynthesis is impaired [1]. Considering this, iron-depletion resulted in a paradoxical depression of the polyamine importer, SLC22A16, in SK-Mel-28 cells (Fig. 6A), but not MCF-7 cells (Fig. 6B). Thus, we next examined the effect of iron on the transport activity of the polyamine uptake system.

To assess polyamine uptake, cells were incubated for 24 h at 37°C under our standard conditions with control medium, or this medium containing 311 (25 μM), DFO (100 μM), or FAC (100 μg/mL). Additionally, the ODC inhibitor, DFMO (5 mM), was added and incubated with either control medium, 311, DFO, or FAC for 24 h at 37°C. The effects of DFMO were assessed as this agent leads to inhibition of ODC activity [72, 73]. This results in decreased polyamine levels that induces decreased Antizyme translation, and thus, relieving the repression on polyamine uptake [1, 73]. Therefore, the effects of iron-depletion in the context of incubation with DFMO was examined. Following these treatments, cells were incubated with $^3$H-spermidine (1 μM) for 1 h at 37°C, after which they were washed and internalized $^3$H-spermidine determined (Fig. 7C, D).

First, there was a marked differential in $^3$H-spermidine uptake between the cell-types after iron-depletion (Fig. 7C, D) that corresponded to levels of the polyamine importer, SLC22A16 (Fig. 6A, B). In fact, iron-depletion by 311 or DFO resulted in a marked and significant ($p < 0.01$) reduction in 17 internalized $^3$H-spermidine uptake to < 50% of the vehicle control in SK-Mel-28 cells (Fig. 7C), while there was no significant ($p > 0.05$) effect of these agents in MCF-7 cells (Fig. 7D). Incubation of cells with FAC did not cause a significant change in $^3$H-spermidine uptake relative to the control (data not shown), which corresponded to the lack of alteration in SLC22A16 levels in response to this agent (Fig. 6B).

DFMO irreversibly inhibits ODC activity [20]. However, by decreasing ODC activity, DFMO also elicits a compensatory increase in the uptake of exogenous polyamines via a mechanism involving decreased Antizyme expression [20]. This homeostatic response to ODC inhibition is why DFMO failed to show clinical efficacy in various cancer-types [73]. Consistent with this response to DFMO, incubation of both cell-types with DFMO in control media led to a significant ($p < 0.05$) increase in internalized $^3$H-spermidine uptake relative to the control (Fig. 7C, D). Examining both cell-types, 311 or DFO suppress the DFMO-induced increase in $[^3$H]-spermidine uptake to similar levels observed under control conditions with these chelators (Fig. 7C, D). This observation indicates that iron-depletion leads to decreased polyamine uptake, even in the presence of DFMO. In summary, $^3$H-spermidine uptake was strongly associated with the iron-dependent regulation of the polyamine transporter, SLC22A16 (Fig. 6A, B).

3.9 Iron modulates expression of the key up-stream polyamine regulators, c-Myc and p53

To assess the mechanism(s) by which iron-depletion “reprograms” polyamine metabolism, the effects of modulating iron on the expression of key up-stream drivers of the polyamine pathway were examined. Specifically, we examined the regulation of key transcription
factors, namely the proto-oncogene, c-Myc, and the tumor suppressor, p53, which are involved in modulating: ADI1, the ODC-Antizyme-AZIN1 axis, and SAT1 [17, 74–78] (Fig. 8A, B).

Notably, c-Myc is a predicted transcriptional activator of ADI1 [76], and a major transcriptional driver of ODC[17]. Moreover, c-Myc is down-regulated by iron-depletion [79, 80]. On the other hand, the tumor suppressor, p53, may act as a transcriptional repressor of ODC[74], and p53 positively regulates polyamine catabolism at the level of increasing SAT1 expression [75]. Cellular iron-depletion up-regulates p53 protein and DNA-binding activity in MCF-7 cells that express wild-type p53 [31, 81]. As such, we hypothesized the ability of iron levels to regulate polyamine metabolism via the ODC-Antizyme-AZIN1 axis, as well as via the rate-limiting catabolic enzyme, SAT1, would depend on the regulation of c-Myc and p53.

To assess this hypothesis, we first examined the ability of iron to regulate c-Myc and p53 expression. As above, cells were incubated with control medium, or this medium containing either: 311 (25 μM), DFO (100 μM), or the iron donor, FAC (100 μg/mL), for 24 or 48 h/37°C. Protein expression was then assessed by western analysis (Fig. 8A, B). In both cell-types, incubation with 311 or DFO, led to a significant (p < 0.001) increase in the iron-regulated positive control, TfR1. When c-Myc expression was examined, a significant (p < 0.001) decrease in expression was observed in response to iron-depletion for both cell-types at both 24 and 48 h (Fig. 8A, B). In contrast, p53 levels were significantly (p < 0.001) decreased only in SK-Mel-28 cells (Fig. 8A), while they were significantly (p < 0.001) increased in MCF-7 cells (Fig. 8B). Considering these results, MCF-7 cells possess wild-type p53, which is capable of tetramerization, nuclear translocation and transcriptional activity [82]. On the other hand, SK-Mel-28 cells express a missense mutant p53 (i.e., p.L145R; COSMIC Database [83]) that is devoid of these latter activities. In fact, p53 appears non-functional in SK-Mel-28 cells, as it fails to promote cell cycle arrest and apoptosis [84].

As c-Myc [17] and p53 [74] are opposing regulators of ODC transcription, we also examined the regulation of ODC mRNA in response to iron in both cell-types after a 48 h incubation (Suppl. Fig. 1). As discussed above, 311 and DFO decreased the mRNA levels of ODC in SK-Mel-28 cells (mutant p53; Suppl. Fig. 1A), despite increased ODC protein after a 48 h incubation (Fig. 3A). The increase in ODC protein, despite the decreased ODC mRNA in SK-Mel-28 cells, may be due to the decrease of Antizyme and increase of AZIN1 (see Fig. 3A).

Examining MCF-7 cells (wild-type p53), incubation with 311 or DFO also resulted in a significant (p < 0.001 – 0.05) decrease in ODC mRNA levels (Suppl. Fig. 1B), despite no significant (p > 0.05) change in ODC protein levels after a 24 or 48 h incubation (Fig. 3B). The iron-depletion-dependent decrease in ODC mRNA in MCF-7 cells observed here (Suppl. Fig. 1B), and in our previous studies [14], as well as by others [13], is consistent with the decrease in the ODC transcriptional activator, c-Myc, after chelation (Fig. 8B) [31, 81]. Additionally, after iron depletion, there is an increase in the ODC transcriptional repressor, p53 (Fig. 8B), which is wild-type in MCF-7 cells [82]. This could 19 potentiate
the down-regulation of _ODC_ mRNA in these cells (Suppl. Fig. 1A,B) together with the decline of c-Myc after iron-depletion (Fig. 8B).

### 3.10 Silencing of c-Myc alters iron-dependent changes in key polyamine pathway proteins

The roles of c-Myc and p53 in regulating the effects of iron-depletion on ADI1, the ODC-Antizyme-AZIN1 axis and SAT1 were examined by assessing expression of relevant polyamine pathway proteins after silencing of _c-Myc_ or _p53_, relative to control, non-targeting siRNAs (Figs. 9, 10A, B). In these studies, siRNA pools specific for _c-Myc_ or _p53_ were compared to a non-targeting siRNA pool (_siNC_) and incubated with cells for 48–72 h. The cells were then incubated with control (media), 311 (25 µM), DFO (100 µM), or FAC (100 µg/mL) for a further 48 h/37°C. The polyamine pathway proteins examined were the iron-containing methionine salvage enzyme, ADI1, as well as ODC, Antizyme and AZIN1, which are responsive to, or predicted to be responsive to [e.g., ADI1 [76]], changes in _c-Myc_ [17, 77] and _p53_ expression [74, 78]. We also examined SAT1, which is a _p53_-activated target-protein [75]. Collectively, _p53_ may be capable of down-regulating polyamine levels by suppressing _ODC_ and increasing _SAT1_ expression.

When cells were transfected with _c-Myc_ siRNA (_si-Myc_), compared to _siNC_, _c-Myc_ levels were significantly (_p < 0.001 – 0.05_) suppressed in SK-Mel-28 (Fig. 9A) and MCF-7 cells (Fig. 9B). The silencing of _c-Myc_ in SK-Mel-28 cells had the following effects relative to the _siNC_ (Fig. 9A): _i._ a significant (_p < 0.001_) decrease in TfR1 in Control, 311 and DFO-treated cells, which is consistent with the known role of _c-Myc_ in the transcriptional activation of _TfR1_ [77]; _ii._ a significant (_p < 0.001 – 0.05_) decrease in ADI1 protein for all conditions (except DFO), which is consistent with the existence of _c-Myc_/MAX binding sites in the _ADI1_ promoter [76]; _iii._ a significant (_p < 0.001 – 0.05_) decrease in ODC protein for all _si-Myc_-treated conditions, which is consistent with the transcriptional activation of _ODC_ by _c-Myc_ [17, 77]; _iv._ a significant (_p < 0.01 – 0.05_) decrease in AZIN1 levels for all conditions (except DFO) in _si-Myc_-treated cells; and _v._ a significant (_p < 0.001 – 0.01_) decrease in Antizyme expression under all _si-Myc_-treated conditions.

Similarly to the effect on SK-Mel-28 cells (Fig. 9A), _c-Myc_ silencing in MCF-7 cells (Fig. 9B) resulted in a significant (_p < 0.001 – 0.01_) decrease in TfR1 in Control, 311 and DFO-treated cells relative to the respective _siNC_ treatments. Further, as also found for SK-Mel-28 cells (Fig. 9A), _c-Myc_ silencing in MCF-7 cells led to a significant (_p < 0.001 – 0.01_) decrease in ODC protein relative to all respective incubation conditions after _siNC_ treatment (Fig. 9B). Again, this is consistent with the transcriptional activation of _ODC_ by _c-Myc_ [17, 77]. The silencing of _c-Myc_ also led to key differences in the expression of polyamine pathway proteins relative to the _siNC_ condition (Fig. 9B), namely: _i._ a pronounced and significant (_p < 0.001_) decrease in ADI1 protein levels in Control and FAC-treated cells; _ii._ a significant (_p < 0.001 – 0.01_) increase in Antizyme in the control and FAC-treated samples after _si-Myc_-treatment; and _ii._ a marked and significant (_p < 0.001) increase in AZIN1 after _c-Myc_ silencing, but only following iron-depletion with 311 or DFO.

Collectively, these data indicate that _c-Myc_ is a regulator of the ODC-Antizyme-AZIN1 axis in both cell-types. Moreover, _c-Myc_ is a modulator of the effects of iron-depletion on this
crucial biosynthetic axis of the polyamine pathway in SK-Mel-28 and MCF-7 cells. In particular, the decreased ODC expression after silencing the pro-proliferative proto-oncogene, \( c-Myc \), would lead to decreased polyamine levels (Fig. 7A, B) that would reduce growth.

### 3.11 Silencing of p53 alters iron-dependent changes in key polyamine pathway proteins

As p53 levels were differentially regulated by iron-depletion in SK-Mel-28 and MCF-7 cells (Fig. 8A, B), we assessed the effects of p53 silencing on ADI1, and the above regulatory proteins of the ODC-Antizyme-AZIN1 axis (Fig. 10A, B). We also assessed the effect of p53 silencing on SAT1, a p53-activated protein [75].

When cells were transfected with \( p53 \) siRNA (i.e., \( si-p53 \)), compared to the relevant non-targeting siRNA (\( siNC^* \)), p53 levels were generally markedly and significantly \((p < 0.001 – 0.05)\) reduced in SK-Mel-28 cells (Fig. 10A) and MCF-7 cells (Fig. 10B). The silencing of \( p53 \) in SK-Mel-28 cells did not significantly \((p > 0.05)\) alter the expression of TIR1, ADI1, ODC or SAT1. This effect is probably because SK-Mel-28 cells possess a non-transcriptionally-active missense mutant of p53 [83, 84]. In contrast to the above proteins, the silencing of mutant p53 in SK-Mel-28 cells led to a significant \((p < 0.05)\) decrease in Antizyme, but only in cells incubated with 311 or DFO (Fig. 10A). Moreover, silencing of mutant p53 also led to a significant \((p < 0.001 – 0.05)\) increase in AZIN1, but only in cells incubated with 311, DFO or FAC (Fig. 10A). Our results suggest mutant p53 in SK-Mel-28 cells can regulate Antizyme and AZIN1 levels, but the mechanism by which this occurs is unknown.

When assessing MCF-7 cells, the silencing of wild-type \( p53 \), which these cells are known to possess [82], led to a significant \((p < 0.001)\) decrease in TIR1, but only following iron-depletion (Fig. 10B). Although ADI1 is not known to be regulated by p53, the silencing of p53 led to a significant \((p < 0.001)\) decline in ADI1 in control cells and cells that had been incubated with FAC (Fig. 10B). In the case of ODC, despite the transcriptional suppression of ODC by p53 in other cell-types [74, 78], there was no significant \((p > 0.05)\) change in ODC protein when \( p53 \) was silenced in MCF-7 cells, except after incubation of cells with 311 (Fig. 10B). In this latter case, ODC expression was significantly \((p < 0.001)\) decreased to \(-60\%\) (Fig. 10B).

Silencing of \( p53 \) in MCF-7 cells led to a significant \((p < 0.05)\) decrease in Antizyme levels, and a significant \((p < 0.001 – 0.05)\) increase in AZIN1 following iron-depletion with 311 or DFO (Fig. 10B). This latter response resembles the effects of mutant \( p53 \) silencing on Antizyme and AZIN1 expression in SK-Mel-28 cells, and again, suggests p53 acts to promote Antizyme and suppress AZIN1 expression.

We next examined SAT1, the major rate-limiting enzyme of polyamine catabolism and a transcriptional target of p53 [75]. The up-regulation of SAT1 in response to chelators in \( siN9C^* \)-treated MCF-7 cells could be due to up-regulation of p53, as these cells possess wild-type \( p53 \) [83, 84], which is up-regulated by iron-depletion (Fig. 10B). However, as p53 is mutant in SK-Mel-28 cells [83, 84], and is down-regulated after iron-depletion (Fig. 8A), it is likely that a different mechanism is involved in SAT1 up-regulation in this latter cell-
type. Thus, SAT1 levels were also assessed when p53 was silenced in both cell-types (Fig. 10A, B). Silencing mutant p53 in SK-Mel-28 cells led to no significant (p > 0.05) change in SAT1 levels relative to the siNC* (Fig. 10A). However, silencing of wild-type p53 in MCF-7 cells led to a significant (p < 0.001) decrease in SAT1 levels after iron-depletion by 311 and DFO (Fig. 10B). These results support the hypothesis that SAT1 is regulated by p53, and the up-regulation of SAT1 in these cells by iron-depletion is partly dependent on p53.

Together, the results herein indicate involvement of c-Myc and/or p53 in mediating some key iron-depletion-dependent changes in key proteins involved in the biosynthetic and catabolic arms of the polyamine pathway in SK-Mel-28 and MCF-7 cells: namely, ADI1 and SAT1.

3.12 Supplementation with spermidine and putrescine rescues the iron chelation-mediated decrease in proliferation

Iron-depletion regulates the expression of 13 major polyamine pathway proteins (Figs. 2–6), as well as decreasing intracellular spermidine and/or spermine levels (Fig. 7A, B). Considering that both iron and polyamines are required for proliferation, with iron-depletion inhibiting proliferation [46, 47, 52–55], studies examined if this detrimental effect could be “rescued” by supplementation with exogenous polyamines (Fig. 11).

To assess effects on proliferation, SK-Mel-28 and MCF-7 cells were incubated for 72 h/37°C with control medium or medium containing 311 (0.02 – 25 µM), DFO (0.1 – 100 µM), and either putrescine, spermidine, or spermine (100 µM or 1 mM). These relatively high polyamine concentrations were employed as tools to overcome the deficits in polyamines that occurs following iron-depletion (Fig. 7A, B). Such a polyamine replenishment approach has been used to demonstrate a requirement for polyamines in other processes [85]. The concentrations of chelators required to inhibit proliferation by 50% of the control over 72 h were then calculated (i.e., IC_{50} values). The effects of exogenous polyamines on these IC_{50} values were then examined.

Notably, the bovine serum amine oxidase (BSAO) inhibitor, aminoguanidine, is usually added in such experiments to prevent BSAO oxidizing exogenous polyamines that produces toxic aldehydes and hydrogen peroxide [86–88]. However, aminoguanidine was avoided due its possible anti-proliferative activity [89]. Indeed, these studies were designed to assess the ability of exogenous polyamines to rescue the inhibition of proliferation by chelators, which would have been confounded by the anti-proliferative activity of aminoguanidine. Moreover, and as described below, the addition of exogenous polyamines in these “rescue experiments” did not lead to cytotoxicity and, under the majority of conditions, prevented iron-depletion mediated anti-proliferative activity in serum-containing media (Fig. 11). This may be due to the ability of the chelators to remove copper from BSAO, inhibiting its ability to generate toxic aldehydes and hydrogen peroxide.

As shown previously, DFO and 311 are well-characterized iron chelators with their anti-proliferative activity due to their ability to bind iron pools, diminishing metabolic utilization of this metal [15, 50, 52, 55]. Moreover, their anti-proliferative activity is prevented by the addition of iron salts, demonstrating their activity is due to iron chelation [40]. In the current
studies, 311 demonstrated significantly \((p < 0.001)\) greater anti-proliferative activity \(i.e.,\) lower IC\(_{50}\) than DFO in both cell-types (Fig. 11A, B). This enhanced efficacy of 311 is probably due to the markedly greater lipophilicity of this agent relative to DFO, leading to the increased iron chelation efficacy \[15, 50, 52\].

In terms of the rescue-efficacies of the three major polyamines in preventing the chelator-mediated inhibition of proliferation, differential effects were observed between both cell-types. Putrescine and spermidine demonstrated the greatest ability to ameliorate the anti-proliferative efficacy of DFO and 311 (Fig. 11A, B). This rescue effect was dose-dependent, with the greatest increase in chelator IC\(_{50}\) occurring at putrescine and spermidine concentrations of 1 mM for SK-Mel-28 and MCF-7 cells (Fig. 11A, B).

The effect of putrescine in rescuing the anti-proliferative activity of iron-depletion is noteworthy, as putrescine levels were not affected in either SK-Mel-28 or MCF-7 cells after incubation with DFO or 311 (Fig. 7A, B). However, AMD1 levels were significantly elevated by iron-depletion in SK-Mel-28 and MCF-7 cells after 48 h (Fig. 2A, B).

Considering this, and as AMD1 is a major rate-limiting step for spermidine and spermine production, under these conditions, putrescine would be converted to spermidine and spermine by SRM and SMS, respectively (Fig. 1). This may restore the levels of these latter polyamines, which were decreased by iron-depletion (Fig. 7A, B), to rescue proliferation.

The ability of spermidine to rescue the anti-proliferative activity of chelators \(i.e.,\) increase the chelator’s IC\(_{50}\) value was less pronounced for 311 relative to DFO for SK-Mel-28 (Fig. 11A) and MCF-7 cells (Fig. 11B). This is probably because of the marked anti-proliferative efficacy of 311 \[15, 50, 52\]. However, the reverse was true for putrescine in SK-Mel-28 cells, which was more effective in rescuing the anti-proliferative activity of 311 relative to DFO (Fig. 11A). In contrast, examining MCF-7 cells, putrescine showed similar activity at rescuing proliferation after incubation with 311 or DFO (Fig. 11A). When DFO was incubated with SK-Mel-28 cells, spermidine (1 mM) was more effective at rescuing the anti-proliferative effect of this chelator than putrescine (Fig. 11A). Again, a similar, albeit less pronounced trend, was observed for MCF-7 cells treated with DFO and putrescine or spermidine (Fig. 11B).

Considering the effects of spermine, this polyamine demonstrated a much less pronounced capacity to rescue the anti-proliferative effects of the chelators (Fig. 11A, B). Moreover, for SK-Mel-28 cells, the combination of spermine and DFO resulted in an enhancement, rather than a rescue, of the anti-proliferative effects of this chelator (Fig. 11A). This potentiation of DFO’s anti-proliferative activity by spermine may be due to up-regulation of the H\(_2\)O\(_2\)- and aminoaidehyde-producing enzyme, SMOX, in SK-Mel-28 cells, but not MCF-7 cells (Fig. 5A). In SK-Mel-28 cells, the oxidation of high levels of exogenous spermine by the increased SMOX levels may enhance the anti-proliferative activity of excessive enzymatic and cytotoxic reaction products, namely hydrogen peroxide or aminoaidehlydes \[90\].

Collectively, these data support the hypothesis that a significant component of the anti-proliferative activity of iron-depletion can be rescued by supplementation of putrescine and
spermidine. This suggests that dysfunctional polyamine metabolism contributes to the antiproliferative effects of iron-depletion.

4. Discussion

Herein, we demonstrate that iron levels significantly altered the expression of 13 major polyamine pathway proteins, which are involved in the biosynthesis, catabolism and transport of polyamines, across two cell-types (Figs. 2–6). Seven of these proteins were regulated similarly by iron levels (i.e., “conserved changes”), with four proteins being down-regulated (i.e., ADI1, MAT2α, Antizyme, PAOX) and three proteins being up-regulated (i.e., AMD1, SAT1 and AZIN1). Interestingly, other than SAT1 and PAOX, polyamine catabolic and transport proteins were differentially modulated between the cell-types. Overall, these data demonstrate that polyamine metabolism (Fig. 1) is regulated by iron-depletion and resulted in decreased polyamine levels. Considering that iron is indispensable for growth [91], these findings indicate polyamine metabolism is coordinated by iron levels.

4.1 Conserved Alterations in Seven Major Polyamine Proteins Between Cell-Types

Regarding the conserved changes in seven major polyamine pathway proteins (i.e., ADI1, MAT2α, AMD1, Antizyme, AZIN1, SAT1 and PAOX) in response to iron-depletion, it is important to consider: (1) those changes that caused the down-regulation of polyamine pools; and (2) those alterations occurring as a response to decreased polyamines. As such, the alterations in AMD1, Antizyme and AZIN1 are indicative of the homeostatic response to a decline in polyamines [1]. That is, a reduction in polyamines leads to increased AMD1 and AZIN1 expression, and decreased Antizyme, thereby acting to increase polyamine biosynthesis by elevating the two rate-limiting steps in polyamine generation (i.e., dcAdoMet and putrescine syntheses via ODC) to rescue polyamine levels [1].

Of direct relevance to the mechanism-of-action by which iron-depletion decreased polyamine pools, the remaining conserved changes in response to iron-depletion were: (i) significant decreases in ADI1 and MAT2α. (as well as a pronounced decrease in the MAT2α product, AdoMet); and (ii) a significant increase in SAT1. The decline in ADI1 and MAT2α would decrease the capacity for spermidine and/or spermine biosynthesis, while the increase in SAT1 could deplete spermidine and/or spermine pools by promoting their acetylation and downstream catabolism (Fig. 1).

Considering further the effect of iron-depletion on polyamine catabolism, PAOX converts spermine to spermidine and spermidine to putrescine, which is more easily excreted from cells [1]. However, PAOX is constitutively expressed, and therefore, its levels are not typically regulated in response to polyamine levels [1]. Thus, the observation that iron-depletion decreased PAOX in both cell-types suggests it was down-regulated as a response to conserve polyamines upon polyamine-depletion. However, in contrast, the finding that iron-depletion increased SAT1 expression would promote the initial phase of polyamine catabolism, in which N₁-acetylspermine or N₁-acetylspermidine are synthesized. Thus, iron-depletion may disrupt the integration of polyamine catabolism via the SAT1-PAOX couple, which could be another factor leading to inhibition of proliferation.
4.2 Cell-Type Dependent Differences in ODC Regulation by Iron-Depletion

Beyond the conserved changes in seven polyamine metabolic proteins, there were cell-type-specific differences in regulation of the remaining six polyamine metabolism proteins examined. In this case, iron-depletion differentially regulated ODC expression, the key rate-limiting step in putrescine production [20]. That is, ODC was up-regulated by iron-depletion in SK-Mel-28 cells, while remaining unaltered in MCF-7 cells. While the regulation of ODC is complex, with multiple levels of regulation [20], ODC expression and/or activity is regulated in response to polyamine levels by altering Antizyme and AZIN1 expression [20] (Fig. 1).

The decrease in ODC expression in response to binding of ODC by Antizyme is mediated by proteasomal activity [20]. Thus, for SK-Mel-28 cells, for which ODC mRNA (Suppl. Fig. 1) was reduced in response to iron-depletion, while ODC protein was concomitantly increased (Fig. 3A), this response can be explained, in part, by the decrease in Antizyme and increase in AZIN1 (Fig. 3A). That is, such changes would act to increase ODC protein-expression at the post-translational level. In this case, the extent of the increase in ODC protein by this latter mechanism appeared to predominate over any decrease in protein translation mediated by a reduction in ODC mRNA expression, leading to a net increase in ODC protein (Fig. 3A).

In contrast, while ODC mRNA levels were also reduced in MCF-7 cells upon iron-depletion (Suppl. Fig. 1), ODC protein expression was unaffected at 24 and 48 h (Fig. 3B). This response may be explained by the extent of the increase in ODC protein mediated by the Antizyme-AZIN axis in MCF-7 cells, which may have been counteracted by the decrease in ODC mRNA levels after iron-depletion, leading to no change in ODC protein.

Cell-type differences in the regulation of ODC are not unusual, as polyamine metabolism is dictated by the specific metabolic characteristics of different cell-types, including different tumor-types [1, 10, 20]. These metabolic differences are modulated by the different molecular phenotypes frequently observed in cancer cells [1, 10, 20, 68]. Indeed, this is demonstrated herein for c-Myc and p53, which are regulated by iron levels and act antagonistically to affect the expression of key polyamine pathway proteins (Figs. 8–10).

4.3 Reprogramming Polyamine Metabolism by Iron-Depletion shows some Dependence on Iron-Regulated Expression of c-Myc and p53

The current studies demonstrate the iron-dependent regulation of c-Myc and p53 (Fig. 8) results in antagonistic effects on polyamine pathway protein expression (Figs. 9, 10). This is probably related to the roles of these proteins in stimulating and inhibiting proliferation, respectively [74, 77]. For instance, the decreased ODC expression observed after silencing pro-proliferative c-Myc, would decrease polyamines that reduces proliferation. Of note, c-Myc is a major transcriptional driver of ODC expression [17], and the reduction in c-Myc after iron-depletion in both cell-types (Fig. 8A, B) probably plays a role in the decrease of ODC mRNA in SK-Mel-28 and MCF-7 cells (Suppl. Fig. 1). However, for both cell-types, ODC protein levels were either increased (SK-Mel-28; Fig. 3A), or not altered (MCF-7; Fig. 3B) over the 24 h or 48 h incubations examined. This indicates the complex regulation of
ODC and the effects of other regulatory mechanisms, such as the Antizyme-AZIN1 axis [20] and predicted changes in proteasomal activity resulting from p53 missense mutant-status [92]. We also observed a decreased ADI1 expression after silencing the transcriptional activator of ADI1 transcription, c-Myc [76]. These results suggest the down-regulation of ADI1 by iron-depletion may involve decreased c-Myc-mediated transcription of ADII.

In addition to the above mechanisms, the regulatory effect of the tumor suppressor, p53, on ODC must also be considered. In contrast to c-Myc, wild-type p53 can act as a transcriptional repressor of ODC [74]. Further, p53 positively regulates polyamine catabolism by increasing SAT1 expression [75]. Hence, the overall effect of wild-type p53 would be to decrease polyamines and inhibit growth. However, these effects of wild-type p53 on polyamine metabolism are complicated by p53 mutations, which in the case of SK-Mel-28 cells, result in its failure to promote cell cycle arrest and apoptosis [84]. This lack of transcriptional activity of mutant p53 is reflected by the fact that silencing mutant p53 in SK-Mel-28 cells showed little effect on the expression of the key polyamine pathway enzymes that it regulates, particularly ODC [74] and SAT1 [75] (Fig. 10A).

Moreover, silencing wild-type p53 in MCF-7 cells in the presence of iron-depletion also had little effect on ODC expression (Fig. 10B). This was despite the down-regulation of Antizyme and up-regulation of AZIN1, which would promote an increase in ODC (Fig. 1). In contrast to SK-Mel-28 cells, the effects of iron chelation were to markedly up-regulate wild-type p53 in MCF-7 cells (Figs. 8B, 10B). This should have an opposite effect to p53 siRNA, namely to down-regulate AZIN1 and up-regulate Antizyme, that would inhibit ODC expression. However, examining MCF-7 cells, despite the up-regulation of p53 after iron chelation, ODC levels remained the same as the control. Such differences between the two cell-types may be explained, in part, by the differential effects of p53 mutant-status on proteasomal activity [92].

The complexity of regulating ODC at multiple levels by iron-depletion could be mediated by at least three mechanisms. These include: (1) the AZIN1-Antizyme axis, whose expression after iron-depletion in both cell-types (Fig. 3A, B) would act to increase ODC due to the decrease in polyamines (Fig. 7A, B); (2) the pro-proliferative oncogene, c-Myc, which is down-regulated after iron-depletion in both cell-types (Fig. 8A, B) and could act to reduce ODC expression; and (3) at least for MCF-7 cells with wild-type p53, the up-regulation of p53 after iron-depletion (Fig. 8B) could be expected to down-regulate ODC.

Examining SK-Mel-28 and MCF-7 cells, neither silencing of wild-type nor mutant p53, respectively, had a pronounced effect on ODC protein levels. In contrast, the silencing of wild-type or mutant p53 resulted in a similar decline in Antizyme and increase in AZIN1 under conditions of iron-depletion in both cell-types (Fig. 10A, B). This regulation of Antizyme and AZIN1 suggests p53 acts to suppress polyamine biosynthesis by restraining polyamine-dependent control of Antizyme and AZIN1 levels. Moreover, as SK-Mel-28 cells possess mutant p53, the mechanism by which p53 regulates Antizyme and AZIN1 cannot require canonical p53-mediated activities.
Assessing SK-Mel-28 cells, the p53 target, SAT1 [75], was not markedly affected by silencing mutant p53. On the other hand, silencing wild-type p53 in MCF-7 cells led to a decrease in SAT1 after iron-depletion by 311 and DFO (Fig. 10B). Since p53 is up-regulated by iron-depletion in MCF-7 cells (Fig. 10B), an increase in SAT1 expression would be expected and was observed in MCF-7 cells (Fig 5B). Hence, the tumor suppressive effects of wild-type p53 can be mediated, in part, by increasing SAT1 expression that is involved in polyamine catabolism, which then reduces proliferation [24, 25].

4.4 Putrescine and Spermidine Rescue Inhibition of Proliferation by Iron Chelators

A striking observation in this investigation was the ability of putrescine and spermidine to rescue the anti-proliferative activity of DFO and 311 in both cell-types (Fig. 11A, B). Considering the effect of putrescine, this polyamine is generated through ODC activity, and its ability in markedly rescuing iron-dependent proliferation indicates its central role in generating spermidine and then spermine [1] (Fig. 1).

In terms of the rescue effect of spermidine, one specific function is its role as a substrate for the covalent modification of the eukaryotic initiation factor 5A (eIF5A) to form hypusine [93]. This protein is required for protein synthesis and promotes formation of the first peptide bond. Thus, hypusine and eIF5A appear to be vital for proliferation [4]. Two enzymes are involved in hypusine formation in eIF5A, namely deoxyhypusine synthase and deoxyhypusine hydroxylase [94]. The latter protein is an iron-containing enzyme that is inactivated by iron chelators and leads to inhibition of protein synthesis [95]. Hence, the rescue of iron-dependent proliferation with putrescine and spermidine is probably related to the fact that putrescine is converted into spermidine, which is vital for hypusine biosynthesis, and thus, protein synthesis [96]. In fact, DFO and especially 311, markedly inhibit protein synthesis in many tumor cell-types, including SK-Mel-28 cells [52].

5. Conclusion

In conclusion, we demonstrate that iron levels act as a potent regulator of the polyamine pathway at multiple levels (Fig. 12), underscoring a missing, fundamental link in understanding how iron modulates proliferation through the coordination of other key metabolic pathways (e.g., polyamine metabolism). The data presented in this study suggest that iron depletion acts to decrease polyamine levels by a conserved mechanism involving the down-regulation of the biosynthetic enzymes, ADI1 and MAT2α, as well as the up-regulation of the rate-limiting catabolic enzyme, SAT1.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

311 2-hydroxy-1-napthaldehyde isonicotinoyl hydrazone

MTT [3-(4, 5- dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium]

ADI1 acireductone dioxygenase 1

AZIN1 Antizyme inhibitor 1

DFO desferrioxamine

dcAdoMet decarboxylated AdoMet

FAC ferric ammonium citrate

SLC22A16 human solute carrier family 22, member 16

IRP iron regulatory protein

MAT2α methionine adenosyltransferase 2α

ODC ornithine decarboxylase

Antizyme ODC Antizyme 1

PAOX polyamine oxidase

AdoMet S-adenosylmethionine

AMD1 S-adenosyl methionine decarboxylase

SMOX spermine oxidase

SMS spermine synthase

SRM spermidine synthase

SAT1 spermidine/spermine-\(N^1\)-acetyltransferase 1

TfR1 transferrin receptor 1

References


[35]. Lane DJR, Saletta F, Suryo Rahmanto Y, Kovacevic Z, Richardson DR, N-myc downstream regulated 1 (NDRG1) is regulated by eukaryotic initiation factor 3a (eIF3a) during cellular stress caused by iron depletion, PLoS One, 8 (2013) e57273. [PubMed: 23437357]


Figure 1. Schematic illustrating the polyamine metabolic pathway in human cells. This pathway involves three major arms: biosynthesis (blue), which can further sub-divided into: (1) the methionine salvage pathway; (2) the ODC-Antizyme axis; and (3) the “biosynthetic core”; as well as (4) polyamine catabolism (red) and (5) polyamine transport (green).
(1) Methionine Salvage Pathway

Figure 2. The methionine salvage pathway is altered by cellular iron-depletion. (A) SK-Mel-28 or (B) MCF-7 cells were incubated for 24 h or 48 h/37°C with either control medium, or this medium containing 311 (25 μM), DFO (100 μM), or FAC (100 μg/mL). Total protein was then extracted and immunoblotting for TfR1 (positive control for iron-depletion), ADI1, MAT2α, AMD1 and β-actin was performed. Results are typical blots from 3 experiments. The quantitation represents mean ± SD (3 independent experiments). Relative to the respective control: *p < 0.05, **p < 0.01, ***p < 0.001.
Figure 3. The ODC-Antizyme axis is regulated by cellular iron-depletion.

(A) SK-Mel-28 or (B) MCF-7 cells were incubated for 24 h or 48 h at 37°C with either control medium, or this medium containing 311 (25 μM), DFO (100 μM), or FAC (100 μg/mL). Total protein was then extracted and immunoblotting for TfR1 (positive control for iron-depletion), ODC, Antizyme, AZIN1 and β-actin was performed. Results are typical blots from 3 experiments. The quantitation represents mean ± SD (3 independent experiments). Relative to the respective control: *p < 0.05, **p < 0.01, ***p < 0.001.
Figure 4. The “biosynthetic core”, composed of SRM and SMS, is regulated by cellular iron-depletion.

(A) SK-Mel-28 or (B) MCF-7 cells were incubated for 24 h or 48 h/37°C with either control medium, or this medium containing 311 (25 μM), DFO (100 μM), or FAC (100 μg/mL). Total protein was then extracted and immunoblotting for TfR1 (positive control for iron-depletion), SRM, SMS and β-actin was performed. Results are typical blots from 3 experiments. The quantitation represents mean ± SD (3 independent experiments). Relative to the respective control: *p < 0.05, ***p < 0.001.
Figure 5. Polyamine catabolism is regulated by cellular iron-depletion.

(A) SK-Mel-28 or (B) MCF-7 cells were incubated for 24 h or 48 h/37°C with either control medium, or this medium containing 311 (25 μM), DFO (100 μM), or FAC (100 μg/mL). Total protein was then extracted and immunoblotting for TfR1 (positive control for iron-depletion), SAT1, PAOX, SMOX and β-actin was performed. Results are typical blots from 3 experiments. The quantitation represents mean ± SD (3 independent experiments). Relative to the respective control: *p < 0.05, **p < 0.01, ***p < 0.001.
Figure 6. Polyamine transport proteins are regulated by cellular iron-depletion.

(A) SK-Mel-28 or (B) MCF-7 cells were incubated for 24 h or 48 h at 37°C with either control medium, or this medium containing 311 (25 μM), DFO (100 μM), or FAC (100 μg/mL). Total protein was then extracted and immunoblotting for TIR (positive control for iron-depletion), SLC22A16, SLC3A2 and β-actin was performed. Results are typical blots from 3 experiments. The quantitation represents mean ± SD (3 independent experiments). Relative to the respective control: *p < 0.05, ***p < 0.001.
The effect of regulating cellular iron levels on the polyamine content was assessed in: (A) SK-Mel-28 and (B) MCF-7 cells. Cells were incubated for 24 h/37°C with either control medium, or this medium containing 311 (25 μM), DFO (100 μM), or FAC (100 μg/mL). Cells were removed from the substratum, washed and then polyamines were extracted, derivatized and quantitated by HPLC-MS/MS, as described in the Experimental Procedures. Total protein was determined for equivalently treated samples and polyamine levels were
normalized to protein content. The quantitation represents mean ± SD (3 independent experiments). Additionally, the effect of cellular iron-depletion on polyamine uptake was assessed in: (C) SK-Mel-28 or (D) MCF-7 cells. Cells were incubated for 24 h/37°C with either control medium, or this medium containing 311 (25 μM), or DFO (100 μM). Cells were then washed and re-incubated with fresh serum-free medium containing 1 μM ³H-spermidine for 3 h/37°C, washed again, and internalization determined using Pronase (see Experimental Procedures). The quantitation represents mean ± SD (3 independent experiments). In the same experiments in (C) and (D), total cell extracts were prepared and immunoblotting performed for SLC22A16 and β-actin. Results are typical of 3 experiments performed. Relative to the respective control: *p < 0.05, **p < 0.01.
Figure 8. Expression of the key polyamine pathway regulators, c-Myc and p53, is modulated by cellular iron-depletion.

(A) SK-Mel-28 or (A) MCF-7 cells were incubated for 24 h or 48 h/37°C with either control medium, or this medium containing 311 (25 μM), DFO (100 μM), or FAC (100 μg/mL). Total protein was then extracted and immunoblotting for TfR1 (positive control for iron-depletion), c-Myc, p53 and β-actin was performed. Results are typical blots from 3 experiments. The quantitation represents mean ± SD (3 independent experiments). Relative to the respective control: *p < 0.05, ***p < 0.001.
Figure 9. Silencing of c-Myc in (A) SK-Mel-28 cells and (B) MCF-7 cells impacts on iron-dependent regulation of the polyamine pathway.

Cells were transiently transfected for 48 h/37°C with a single siRNA specific for c-Myc (si-Myc) or non-targeting pool (NTP) control siRNA (si-NC), all at a final concentrations of either 5 nM (SK-Mel-28) or 2.5 nM (MCF-7). The cells were then incubated with control medium, or this medium containing 311 (25 μM), DFO (100 μM), or FAC (100 μg/mL) for 48 h/37°C. Total protein was then extracted and expression of TfR1 (positive control for iron-depletion), c-Myc, ADI, ODC, Antizyme, AZIN1 and β-actin were then assessed by immunoblot analysis. Results are typical blots from 3 experiments. The quantitation
represents mean ± SD (3 independent experiments). Relative to the respective control: *p < 0.05, **p < 0.01, ***p < 0.001; Relative to the respective siNC condition: # p < 0.05; ## p < 0.01; ### p < 0.001.
Figure 10. Silencing of p53 in (A) SK-Mel-28 cells and (B) MCF-7 cells impacts on iron-dependent regulation of the polyamine pathway. Cells were transiently transfected for 48 h/37°C with esiRNA pools specific for p53, or non-targeting pool (NTP) control siRNA (siNC*), all at a final concentration of 20 nM. The cells were then incubated with control medium, or this medium containing 311 (25 μM), DFO (100 μM), or FAC (100 μg/mL) for 48 h/37°C. Total protein was then extracted and expression of TfR1, p53, ADI, ODC, Antizyme, AZIN1, SAT1 and β-actin were then assessed by immunoblot analysis. Results are typical blots from 3 experiments. The quantitation represents mean ± SD (3 independent experiments). Relative to the respective
control: $p < 0.05$, $**p < 0.01$, $***p < 0.001$; Relative to the respective siNC* condition: $# p < 0.05$; $## p < 0.01$; $### p < 0.001$. 
Figure 11. Supplementation with exogenous putrescine or spermidine rescues the proliferation of iron-depleted cells.

(A) SK-Mel-28 and (B) MCF-7 cells were incubated for 72 h/37 °C with 311 (0.02–25 μM) or DFO (0.1–100 μM) in the absence or presence of putrescine (100 μM or 1 mM), spermidine (100 μM or 1 mM), or spermine (100 μM or 1 mM). Cellular proliferation was measured using the MTT proliferation assay and IC₅₀ values were then determined. Results are typical of three independent experiments with data analysis representing mean ± SD (n =
3). Relative to the respective 311- or DFO-treated control: \(*p < 0.05, **p < 0.01, ***p < 0.001.\) Relative to the DFO-treated control: \(###p < 0.001.\)
Figure 12. Schematic summarizing the “reprogramming” of polyamine metabolism by iron-depletion in: (A) SK-Mel-28 human melanoma and (B) MCF-7 human breast cancer cells. Iron depletion leads to: (1) alterations in the expression of c-Myc and p53, where iron-depletion decreases c-Myc expression in both cell-types, while decreasing expression of mutant p53 (SK-Mel-28) and increasing wild-type p53 (MCF-7); (2) changes in the expression of polyamine pathway proteins involved in catabolism/efflux and biosynthesis/uptake (increased protein expression is marked by red arrows, while decreased protein expression is marked by blue arrows); (3) conserved alterations in the expression of polyamine pathway proteins in SK-Mel-28 and MCF-7 cells, some of which are downstream of the changes in c-Myc and p53; (4) decreases in key polyamine metabolites, AdoMet and spermine, in SK-Mel-28 cells; and decreases in AdoMet, spermidine and spermine in MCF-7 cells; and (5) decreases in proliferation, which can be partially rescued by supplementation with putrescine and spermidine. Collectively, these data demonstrate that cellular iron levels are a potent regulator of polyamine metabolism at multiple levels. These results have crucial ramifications for understanding the integrated role of iron and polyamine metabolism in the proliferation of cancer cells. That cellular iron levels are a potent regulator of polyamine metabolism at multiple levels. These results have crucial ramifications for
understanding the integrated role of iron and polyamine metabolism in the proliferation of cancer cells.