IMPACT OF FERROPTOSIS INDUCTION ON IRON METABOLISM IN CANCER CELLS HARBORING TP53 MUTATIONS

By

LAURIE RENEE THOMPSON

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Oklahoma State University

Stillwater, OK

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Thesis Approved:

Dr. McKale Montgomery

Thesis Adviser

Dr. Stephen Clarke

Dr. Winyoo Chowanadisai

Name: Laurie Thompson

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Abstract: The tumor suppressor gene TP53 is the most commonly mutated gene in human cancer, but mutations in TP53 do not just result in loss of tumor suppressor function, they can also promote cancer progression by altering cellular iron acquisition and metabolism. A newly identified role for wild-type TP53 in the mediation of iron homeostasis and cancer cell survival lies in its ability to protect against ferroptosis, a form of iron mediated cell death. The purpose of this study was to determine the extent to which TP53 mutation status effects iron-mediated cell death in response to ferroptosis induction. Using H1299 cells, which are null for TP53, we generated cell lines expressing either a tetracycline inducible wild-type TP53 gene, or a representative mutated TP53 gene from exemplary “hotspot” mutations in the DNA binding domain (248Q, 273H, 282W, 245S, 249S and 175H). These six mutation types were selected because they represent 25% of all TP53 mutations in human cancer. TP53 mutants 248Q, 273H, 245S, and 249S were more sensitive to ferroptosis than WT TP53. As iron-mediated lipid peroxidation is critical for ferroptosis induction we hypothesized that iron acquisition pathways would be upregulated in mutant TP53 expressing cells. However, only cells expressing the 248Q, 175H, and 245S TP53 mutation types exhibited statistically significant increases in spontaneous iron regulatory protein (IRP) RNA binding activity following ferroptosis activation. Moreover, changes in the expression of downstream IRP targets were inconsistent with the observed differences in sensitivity to ferroptosis. These findings indicate that canonical iron regulatory pathways are bypassed during ferroptotic cell death. These results also indicate that induction of ferroptosis may be an effective therapeutic approach for tumor cells expressing distinct TP53 mutation types.

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CHAPTER I

INTRODUCTION

Iron is a necessary nutrient but it is also crucial to cancer progression due to its essentiality in cell proliferation. However, in excess, iron also has potential to be reactive and cause damage and inflammation. The damage due to iron overload can lead to liver, heart and pancreas damage, and is thought to be a driver of cancer initiation in these tissues[1,2]. Therefore, a better understanding of iron and its regulation, when it comes to cancer progression, could help us develop and improve cancer therapies. Because iron is a crucial, yet potentially toxic nutritional resource, homeostasis is regulated at both the systemic and cellular level.

Important regulators of cellular iron homeostasis are iron regulatory proteins (IRP) and the iron regulatory elements (IRE) to which they bind. IRPs, found in the cytosol of the cell, bind to the IREs on specific regions of mRNAs that encode proteins involved in iron storage (ferritin), uptake (transferrin receptor; TFRC), utilization (mitochondrial aconitase), and export (ferroportin). When the cell is iron deficient, IRPs bind to the IRE in the 5' end of the untranslated regions (UTR) of mRNAs such as ferritin, mitochondrial aconitase, and ferroportin and inhibit their translation. Conversely, binding to IRE in the 3' UTR of mRNA, such as TFRC, promotes transcript stabilization and increased expression[3]. When the cell is iron sufficient, IRPs do not bind IRE which allows for transcription of ferritin and degradation of transferrin receptor mRNA[4].

In cancer, IRP signaling pathways can be corrupted in an effort to acquire sufficient iron to support rapid cell proliferation. For example, IRP2 overexpression in breast cancer results in increased TFRC expression, decreased ferritin expression, and subsequently an increased labile iron pool[5]. Increased expression of TFRC was also found to have worse clinical prognosis in patients that had renal cell carcinoma[6]. As mentioned above, increased expression of TFRC is typically mediated by increased IRP RNA binding activity, but overexpression of IRP1 was actually found to decrease tumor growth *in vivo*[7]. Thus, the opposing effects of IRP2 overexpression appear to be independent of the influence of IRP2 on iron metabolism and continued investigation into the roles that IRPs play cancer progression is warranted [8].

TP53 is called the "guardian of the genome" for good reason. Under cellular stress, it has the potential to protect and regulate cell life by pausing cell cycle progression to repair DNA or inducing apoptosis. TP53 also regulates autophagy, metabolism, and angiogenesis, and has recently been found to be involved in iron homeostasis[9,10]. For example, TP53 has the ability to modulate IRP1 RNA binding activity through the transcriptional regulation of the iron sulfur cluster assembly enzyme (ISCU)[11]. When an iron sulfur cluster is inserted into IRP1, it cannot bind to the IRE resulting in increased in ferritin protein expression[4]. The ability of TP53 to increase ferritin expression promotes the sequestration of iron into storage, thereby decreasing its availability for use and reactivity, and protecting against cancer progression.

Another newly identified role for TP53 in the mediation of iron homeostasis and cancer cell survival lies in the ability for TP53 to protect against ferroptosis, a form of iron mediated cell death. Ferroptosis is characterized by glutathione (GSH) depletion and iron accumulation resulting in increased lipid peroxidation and oxidative stress[12]. Increased iron uptake is essential for ferroptotic cell death as co-treatment with an iron chelator is sufficient to inhibit ferroptosis induction. Moreover, it is understood that iron uptake via TFRC is necessary for iron-mediated lipid peroxidation but, it is unknown how TFRC is regulated during ferroptosis[13]. Typically,

TFRC expression is modulated by IRPs in response to changes cellular iron status, but IRP binding activity during ferroptosis has not been investigated. Developing an understanding of sensitivity of cells harboring TP53 mutations to ferroptosis could improve therapies targeting specific types of tumors.

Interestingly, wild type TP53 appears to be protective against ferroptosis, while cells with knockout TP53 are more sensitive to ferroptotic cell death[14]. This is important because TP53 mutations are the most common of the genetic alterations in human carcinoma. Somatic mutations occur in 50% of all types of cancers including colorectal and those of the breast, neck, head and many more[15]. When P53 is mutated, it is not able to fully function as it should, which leads to the dysregulation of a multiplicity of pathways regulating cycles in the cell. There are many different types of TP53 mutations but 97% of them are located in the DNA binding domain, and can be categorized into two major groups: contact or conformational[16]. Both mutation types appear in the protein-DNA interface, but contact mutations reside where the protein comes in contact with the DNA without affecting TP53 structure while conformational mutations impact the structure of the core domain of the protein[17-19]. Importantly, the wild-type functions of TP53 are impaired regardless of mutation type, and thus tumors expressing TP53 mutants may be more susceptible to ferroptotic targeting. As TP53 is mutated in more than half of all human cancers, determining the sensitivity of specific TP53 mutation types to ferroptosis activation could have significant implications.

Furthermore, understanding how to manipulate iron metabolism in cancer cells expressing different TP53 mutations could lead to the development of novel therapies. The purpose of this study is to establish how TP53 mutation status effects the iron-mediated cell death in response to ferroptosis induction. We hypothesize that cells harboring TP53 mutations will be more sensitive to ferroptosis than cells expressing wild type TP53. We also hypothesize that IRP RNA binding activity will be further increased in response to ferroptosis activation in cells with

mutated TP53 compared to cells with wild type TP53. To test these hypotheses, we propose these two specific aims:

Aim 1: To determine the extent to which mutant TP53 status influences ferroptotic sensitivity.

Aim 2: To evaluate the impact of ferroptosis on IRP-dependent regulation of iron metabolism.

CHAPTER II

LITERATURE REVIEW

Iron's Impact on Cancer Incidence

Hallmarks of tumor initiation and progression include increased cellular proliferation, deregulated cellular energy metabolism, and vascularization, each of which is dependent upon the acquisition of sufficient levels of iron. Iron also has the capacity to promote damaging free radical production, and thus it has been implicated in tumor initiation as well. Though the mechanisms by which iron contributes to tumor progression and initiation remain poorly described, a significant body of evidence exists suggesting that targeting iron may be a desirable approach for both cancer prevention and treatment. This evidence is underscored by population-based studies that indicate that cancer risk increases with increasing body iron content. For example, patients with hemochromatosis, a genetic disorder causing iron overload, have a significantly higher risk of developing hepatic cancer, with a moderate risk of developing non-hepatic cancers[2,20].

In the general population, there is conflicting evidence as to whether a high dietary intake of iron significantly increased risk of developing cancer. Evaluation of different dietary sources of iron (i.e., heme versus non-heme and food-based versus supplemental) as well as differences in cancer sites likely contribute to conflicting results[2,21,22]. Nevertheless, a large meta-analysis

of dietary intake studies did suggest that higher iron intake is associated with increased risk of colon cancer, regardless of the form in which it was ingested [2,22,23].

Moreover, data from the first NHANES study also indicated a very small increase in transferrin saturation percentage in people that got cancer over a 17 year follow-up period[24]. Likewise, the reduction of iron stores by phlebotomy is associated with a decrease in both cancer-specific and all-cause mortality [25]. It is important to note that iron deficiency can also lead to oxidative DNA damage, which could increase potentially promote cancer initiation, but there is a lack of evidence linking iron deficiency to cancer incidence[26]. Thus, the balancing act between iron toxicity and adequacy are critical for minimizing cancer risk and optimizing health.

Iron Absorption, Transport, Utilization, and Uptake

Iron is an indispensable mineral for mammalian cells due to its essentiality in DNA synthesis, metabolic regulation, cell cycle progression, and cell proliferation[27]. However, free ferrous iron can also create reactive oxygen species through Fenton the reaction, which can initiate lipid peroxidation, oxidative stress, and DNA damage. Thus, iron homeostasis must be highly regulated in order to both avoid deficiency and prevent toxicity. There is no known mechanism for excess iron excretion from mammals, but rather, iron is regulated at the absorptive level in the intestinal enterocyte. Dietary iron, in the form of heme iron enters the enterocyte by a yet unidentified heme transporter. Non-heme iron must first be reduced into a divalent transition metal, Fe^{2+}, by duodenal cytochrome B on the apical membrane, where it then is able to enter the enterocyte via divalent metal transporter 1 (DMT1)[28]. Within the enterocyte iron is either stored by iron storage protein ferritin, used by the enterocyte, or effluxed from the enterocyte by the basolateral membrane protein ferroportin.

Following export across the basolateral membrane, ferrous iron is re-oxidized by hephaestin before being loaded onto the iron transport protein transferrin (TF)[29]. TF plays a

critical role in iron transport because a physiological (neutral) pH iron is insoluble in its free state and can generate free radicals. TF provides a safe means of iron transport, making it available for use by other tissues. TF delivers iron to other tissues via TFRC uptake. TFRC is a membrane glycoprotein that is responsible for iron uptake in all mammalian cells and is involved in cellular regulation of iron metabolism[30]. Once bound, the TF/TFRC complex is endocytosed where the acidic pH of the endosome results in the release of iron. Once in the cell, iron is either stored in ferritin, an iron storage protein, or utilized for energy metabolism and iron containing proteins[31].

Control of Cellular Iron Homeostasis

Iron regulatory proteins (IRPs) control the expression of DMT1, ferroportin, TFRC, and ferritin, and are thus essential controllers of cellular iron homeostasis [4]. IRP1 and IRP2 act as posttranscriptional regulators by binding and releasing from hairpin like structures in the 5' and 3' untranslated regions (UTR) of the mRNA referred to as iron regulator elements (IRE). Under low iron conditions IRP-IRE binding promotes iron uptake, IRP1/2 bind to IREs in the 5' end of the UTRs of transcripts such as ferritin, resulting in translational repression, whereas binding to the IREs in the 3' UTRs of TFRC results in mRNA transcript stabilization[31]. This leads to increased iron uptake and release of stored iron resulting in increased iron availability within the cell.

Under iron replete conditions, IRP1 and IRP2 functionality and regulation differs. IRP1 is a bifunctional protein that functions either as a high-affinity RNA binding protein as described above, or as the cytosolic isoform of the TCA cycle intermediate, aconitase. When sufficient iron is available, insertion of an iron-sulfur cluster into IRP1 prevents it from acting as an RNA binding protein and instead promotes its cytosolic aconitase activity[4,32]. Additionally, IRP1 is also subject to posttranscriptional regulation by other factors such as nitrogen species, reactive

oxygen, phosphorylation and disruptions in iron sulfur (Fe-S) cluster assembly and disassembly[33,34].

Unlike IRP1, IRP2 is regulated via modulation of protein stability and degradation. IRP2 is stabilized during hypoxic and low iron conditions and degraded under iron replete conditions [35,36]. Iron-dependent degradation of IRP2 involves recognition by its E3 ligase, FBXL5, which targets it for ubiquitination and proteasomal degradation[37,38]. Other iron-independent mechanisms for regulation involve nitrogen species, 2-OG-oxygenase, and hypoxia[3,35,36]. Phosphorylation is another mechanism independent of iron that regulates IRP2. This regulation can swap IRP2 from high affinity to low affinity to the RNA[39,40]. The identification of iron-independent pathways by which IRP1 and IRP2 are regulated suggests that extracellular agents capable of orchestrating changes in cellular iron metabolism beyond the canonical alterations in IRP RNA binding activity may still be identified.

The global regulators of iron metabolism, IRP1 and IRP2 seem to have opposing roles in tumors in vivo. In mouse models, IRP2 overexpression promotes tumor growth whereas IRP1 overexpression suppresses tumor growth[8,41]. This is consistent with differing regulation of IRP1 and IRP2. Because IRP2 is usually degraded during iron sufficient conditions, overexpression of IRP2 could lead to inappropriate binding to the IRE resulting in increased iron import via TFRC and decrease iron export via ferroportin[6,42]. This leads to an increase in the labile iron pool, which increase iron's availability for reactivity. These separate findings suggest iron metabolism proteins like ferritin, TFRC, ferroportin and the regulator of these IRP2 have a negative influence on cancer prognosis therefore, influence cancer progression.

Indeed, the iron uptake protein TFRC has is overexpressed in many cancers[43,44]. Increased TFRC expression is also associated with worse tumor pathologies and resistance to chemotherapies and as such has been suggested as a novel biomarker and therapeutic target for

some cancers[30,43,45,46]. As such, anti-TFRC antibodies have been used to inhibit tumor growth[6,30,43,47]. However, because of the systemic effects anti-TFRC antibodies would have on whole body iron homeostasis, the targeting of chemotherapeutic drugs for TFRC-mediated uptake is a more desirable therapeutic approach.

The role of the iron storage protein, ferritin, in cancer progression is not as clear, but perturbations in ferritin levels have been associated with a more malignant phenotype[42]. For example, ferritin regulation depends on the tumor cells tendency toward rapid utilization or storage of iron[48]. Increased ferritin expression has been demonstrated to contribute to growth arrest by restricting iron availability [49]. Conversely, downregulation of ferritin increases the labile iron pool, which can increase tumor cell proliferation[50]. However, the downregulation of ferritin, and subsequently enlarged labile iron pool, is also associated with increase chemotherapeutic sensitivity, presumably by increasing reactive oxygen species production[42,51]. Furthermore, decreased ferritin leads to more iron availability and increase metabolism of tumor cells while decreasing iron storage.

Ferroportin, the iron efflux protein, is decreased in expression in many cancer types, but especially breast cancer. Decreased ferroportin expression is associated with increased levels of the labile iron pool in cultured breast cancer cells and increased growth in breast tumor xenografts[7]. Decreased ferroportin expression was also associated with a poor prognosis in a cohort study of 800 patients with breast cancer[52]. Particularly, decreased ferroportin was associated with increased metastasis, which suggests that ferroportin levels could potentially be used to predict both primary tumor growth as well as metastatic spread. Indeed, an iron-regulatory gene signature has been used to discriminate among breast cancer patients with high versus low risk of distant metastasis free survival[53]. The utility of an iron regulatory gene signature in other cancers has not been described, but given the importance of iron regulatory genes to cancer progression deserves continued investigation.

The Tumor Suppressor TP53

TP53 was first described as an oncogene in 1979 by Lane and Crawford who hypothesized it was an important oncogene of SV40, a oncoprotein tumor virus[54,55]. Soon after, P53 was actually discovered to be a tumor suppressor. This understanding developed from the description of an inherited familial predisposition to cancer development, termed Li-Fraumeni syndrome in 1982[56]. Then in 1990, the primary cause of Li-Fraumeni syndrome was found to be an inherited gene mutation in TP53[57]. After this discovery, the tumor suppressive qualities of P53 were confirmed with a mouse study in which knock out of TP53 lead to tumor development in 100% of the mice[58]. After this establishment, more studies on the genome of tumor cells found that TP53 was mutated more often than any other gene in tumor cells[59]. These genetic studies also found that cells with TP53 mutations could have a "gain of function" which actually results in the development of oncogenic properties.

TP53 is a tetramer protein that has an important role as a potent transcriptional regulator of protein expression. TP53 expression increases in response to stress in normal functioning cells and acts as transcriptional regulator to control the expression of proteins that aid the cell during stress. More specifically,TP53 has been found to be involved in DNA repair, angiogenesis, senescence, metabolism, and autophagy[60]. Usually, when it is latent, during low or absent stress, TP53 is targeted for proteasomal degradation via its E3 ubiquitin ligase, MDM2[61]. Thus, under normal conditions, TP53 protein expression remains low to undetectable[62]. In response to stress, MDM2 does not bind and TP53 protein rapidly accumulates and functions by slowing or stopping the cell cycle to allow the cell to repair DNA damage through transcriptional activation of P21, MDM2 and GADD45[10,62]. If the DNA is beyond repair, TP53 has the ability to induce apoptosis through transcriptional activation of BAX, and IGF-BP[61,62].

The Relationship between Iron and P53

Interestingly, P53 is regulated in response to changes in iron availability, but it can likewise influence cellular iron metabolism and homeostasis. For example, TP53 is destabilized and exported from the nucleus in response to heme supplementation *in vitro*[9]. Conversely, TP53 is stabilized by iron chelators due to hypoxia-inducible factor 1 alpha[63]. Thus, the capacity for iron status to impact TP53 expression suggests a role for iron in cancer development beyond that of ROS production, but also by influencing TP53's tumor suppressive functions.

On the other hand, TP53 was found to be a regulator of iron homeostasis through the transcriptional regulation of the iron regulatory enzyme iron sulfur assembly enzyme (ISCU)[11]. This enzyme acts as a scaffold for the assembly of iron sulfur clusters. Iron sulfur clusters are proteins involved in many functional processes like regulatory, DNA repair, catalyst and electron transfer functions. As IRP1 is also an iron sulfur cluster containing protein, iron sulfur cluster biogenesis can directly influence the control of cellular iron homeostasis. Insertion of an iron sulfur cluster into the RNA binding site of IRP1 prevents its RNA binding activity, which can lead to increased iron storage and decreased iron uptake[64]. Thus, another tumor suppressive function of TP53 is to promote iron sequestration into storage and to reduce iron availability for tumor cell proliferation. Unfortunately, TP53 is mutated in more than half of all cancers, and TP53 mutations correspond with reduced ISCU expression, which could potentially increase the labile iron pool[11].

Interestingly, another enzyme critical for iron sulfur cluster biogenesis, ferredoxin reductase (FDXR) was also recently demonstrated to influence TP53-dependent tumor suppression via IRP2[65]. In this context, knockdown of FDXR increased IRP2 expression and IRP2 decreased TP53 expression via interaction with a putative IRE in the 3' UTR of TP53. This resulted in mitochondrial iron overload and spontaneous tumor development in mice.

Importantly, TP53 overexpression was able to correct the FDXR-mediated abnormalities in iron homeostasis, suggesting that the FDXR-P53 loop is critical for tumor suppression via regulation of iron homeostasis. The influence of TP53 mutations on FDXR-mediated control or iron homeostasis was not investigated, but given the prevalence of TP53 mutations in human cancers, these studies are warranted.

Mutant P53

A gene mutation is an alteration in the DNA sequence that makes up a gene. There are several different types of mutations that can occur including substitution, insertion, deletion and frameshift. Subcategories for mutations involving substitutions are known as silent, nonsense, and missense. Substitution mutation cause a swap in a nucleotide for another. For example, guanine could be exchanged for thymine in a DNA sequence, and thus just one codon would be affected. The difference in substitution subtypes has to do with what effect the mutation has on the protein. Missense leads to an altered codon from an insertion of a different amino acid during protein translation[66]. Another way to characterize mutations is by establishing their origin of change. Germ-line mutations are variation passed on from the germ cells and somatic mutations originate from the body's cells[67].

A mutation can happen in all coding exons of the TP53 gene. It was found that 73% of all mutations in TP53 are missense mutations. This type of mutation is characterized by a substitution of a single amino acid in the proteins primary structure[68]. There is also a high prevalence of mutations in exons 4-9 that encode for the DNA-binding domain of the protein, which interferes with TP53's transcription factor activity[59]. These DNA-binding domain mutations can further be categorized as conformational or contact mutation.

Contact mutations are characterized by missense changes in the amino acids of the protein that comes into contact with the DNA binding domain therefore impairing the

transcriptional activity of the protein without effecting its overall protein structure or conformation. Conformation mutations on the other hand both interfere with normal DNA binding and alter the proteins overall structure. Though hundreds of P53 mutations identified, 6 sites within the DNA binding domain are mutated at a much higher frequency than either other locations. These 6 so-called "hotspots" (R248Q, R249S, R273H, R282W, R175H and G245S) represent nearly 25% of all TP53 mutations[16].

Mutations in TP53 do not simply result in loss of wild type function. Alterations in transcriptional binding partners can result in TP53 mutations that are even more tumorigenic than total loss of TP53 (null TP53). These types of mutations are known as "gain of function" (GOF) mutations[16,69,70]. Several GOF mutants (R175H, R248Q, and R273H) were found to have increased oncogenic function through stimulation of the Warburg effect by facilitating glucose uptake and increasing lactate production[71]. Altering cell metabolism however is just one example of how GOF mutants promote tumorigenesis. GOF properties have shown the ability to affect other oncogenic processes, such as promoting increased cell proliferation, migration, and metastasis, as well as conferring resistance to chemotherapies[16,69-71].

Thus far, therapeutic strategies for targeting mutant P53 have focused on either decreasing mutant TP53 protein stability or restoring its wild-type function. With regards to GOF mutants, destabilization of mutant TP53 could be a promising therapeutic approach because this would decrease their tumorigenic properties. Intriguingly, genetic manipulation restoring TP53's tumor suppressive functions increased apoptosis and decreased tumor cell growth in mice, but pharmacologic reactivation of TP53 in humans is still in its infancy[72,73]. However, because TP53 mutations are often drivers of chemotherapy resistance, further investigation of therapeutic strategies for targeting TP53 mutants could have significant clinical implications[74].

Ferroptosis

Ferroptosis is a newly described mode of non-apoptotic cell death that is dependent on iron for induction. In order for this type of cell death to take place there needs to be a depletion of glutathione, an inactivation of glutathione peroxidase 4 and an accumulation of free iron (Figure 1)[75-77]. The combination of these characteristics leads to a high level of lipid peroxidation. This type of cell death is separated from apoptosis because it does not require caspase activation. Moreover, unlike apoptosis, iron is essential to ferroptotic cell death because ferroptosis is inhibited cells when iron is depleted by an iron chelator[78].

Figure 1. Mechanisms of ferroptosis induction. Ferroptosis is characterized by lethal levels of lipid hydroperoxides (PL-PUFA(PE)-OOH). Ferroptosis induction and sensitivity are controlled by multiple factors including increased free iron, inactivation of glutathione peroxidase 4 (GPX4), depletion of glutathione and therefore there is an increased in lipid peroxidation ultimately leading to cell death. Other factors involved include amino acids, polyunsaturated fatty acid metabolism, and biogenesis of NADPH, phospholipids (PL-PUFA-PE), and coenzyme Q_{10} (CoQ_{10}). lipoxygenases; ALOXs, 3-hydroxy-3-methylglutaryl-CoA reductase; HMGCR, lysophosphatidylcholine acyltransferse 3; LPCAT3, nuclear receptor coactivator 4; NCOA4, solute carrier family 7 member 11; SLC7A11, squalene synthase; SQS, nuclear factor, erythroid 2 like 2; NRF2. Ferritinphagy is the process of degrading ferritin and releasing the free iron. Image from: Stockwell et al., 2017.

In order for ferroptosis to be induced, the cell needs to accumulate iron via TFRC[13]. As previously discussed, TFRC is regulated by IRPs in response to changes in iron levels, but how TFRC expression is mediated in response to ferroptosis induction has not yet been investigated. Understanding how TFRC expression remains elevated even as the cell becomes saturated with free iron however will be critical to understanding the mechanisms controlling iron-mediated cell death and susceptibility to ferroptosis.

Expression of wild type TP53 has the ability to protect cells from enduring ferroptosis by transcriptionally regulating genes capable of inhibiting lipid peroxidation and slowing glutathione depletion. Stabilization and normal functioning of wild-type TP53 also appears to be necessary for sustained *de novo* glutathione production and increased import of cysteine via system x_c^- which is protective against the lipid peroxidative nature of ferroptosis[14]. Thus, TP53 mutations resulting in loss of wild-type activity can render cells more susceptible to ferroptosis activation. For example, the TP53 mutant expressing colorectal cancer cell lines CACO2 and SW837 are more sensitive to ferroptosis induced by cysteine glutathione antiport system (system x_c^-) via erastin than cells expressing wild-type TP53[79]. This increased sensitivity was demonstrated to result from an inability of mutant TP53 to induce SLC7A11 expression, thereby limiting cysteine uptake and subsequently glutathione production, ultimately leading to ferroptosis[14,70]. However, these studies were not carried out in isogenic cell lines, and thus differences in ferroptotic sensitivity cannot be attributed solely to differences TP53 mutation types. Moreover, the influence of more prevalent "hotspot" TP53 mutations on ferroptosis sensitivity has not been investigated. As these mutation types represent the most prevalent TP53 mutations in human cancers, determining their sensitivity to ferroptosis could have significant implications[16].

CHAPTER III

METHODS

Aim 1: *To determine the extent to which mutant TP53 status influences ferroptotic sensitivity.*

Experimental Methods for Aim 1:

Cell Culture

The human, non-small cell lung carcinoma, H1299 cell line (ATCC CRL-5803) was selected because they do not express an endogenous TP53. For all experiments, cells were grown in RPMI 1640 1X with L-glutamine (Corning #10-040-CV Manassas, VA) supplemented with 10% tetracycline-free fetal bovine serum and 1% penicillin streptomycin 100X (Corning #30-002-Cl Manassas, VA), and maintained in a 37°C incubator with 5% CO_2 and 95% humidity.

Creation of stable TP53 expressing cell lines

Tetracycline inducible plasmids (pcDNA5/TO) containing either wild-type TP53, or a representative "hotspot" TP53 mutant (R248Q, R273H, R175H, R282W, G245S, R245G, or R249S) were generated and validated by GenScript (Nanjing, China). H1299 cells were plated and allowed to grow to 70-90 % confluency in a 6 well plate. Cells were co-transfected with a Tet repressor plasmid, pcDNA6/TR, and one of the tetracycline-inducible plasmids mentioned above

using lipofectamine 3000 reagent (Thermo Fisher Scientific, Waltham, MA Cat # L3000001) according to the manufacturer's instructions. Twenty-four hours after transfection, lipofectamine containing media was removed and replaced with fresh media, and cells were allowed to grow for another 24 hours. Cells were then split to < 25% confluency in media containing both 6 μg/ml blasticidin and 600 μg/ml hygromycin to select for successful transfectants. Following selection, cells were maintained in blasticidin and hygromycin containing media, supplemented with tetracycline-free fetal bovine serum to protect against "leaky" expression. TP53 plasmids were induced by adding10 μg/ml tetracycline to the media for 24 hours.

Western Blot

Western blot was used to validate tetracycline mediated induction of WT TP53 and TP53 mutations inserted into H1299 null cells. Following 24 hours of treatment with 10 μg/ml tetracycline, total protein was harvested by first removing the adherent cells with cell stripper collecting the cells into a microcentrifuge tube. The collected cell suspension was centrifuged at 1,000 x *g* for 5 minutes at 4ºC. The cells were washed with 1X PBS and centrifuged at 1,000 x *g* for 5 minutes at 4ºC. Cells were lysed with RIPA buffer (50 mM Tris-HCl, pH 8.0, 1% NP-40, 0.5% Na-deoxycholate, 0.1% SDS, 2 mM EDTA, 150 mM NaCl, Protease inhibitor cocktail, DTT, Citrate, PMSF, Mg132). Samples were vortexed every 5 minutes for 20 minutes before centrifugation at 14,000 x *g* for 15 minutes at 4ºC. The protein containing supernatant was collected and stored at -80ºC. Total protein concentration was determined based on the bicinchoninic acid assay (Thermo Fisher Scientific, Waltham, MA, Cat #23209). Thirty micrograms total protein was diluted in RIPA and 5X Laemmli sample buffer (LSB) (1% Bromophenol blue, SDS, β-mercaptoethanol, glycerol, Tris-Cl pH 6.8) before heating at 95ºC for 5 minutes to denature the proteins. The protein was then loaded into a Mini-PROTEAN TGX Stain-Free Precast gel with 4-20% polyacrylamide (Biorad Cat #4568096 Hercules, CA) and electrophoresed at 150V for 60 minutes. Protein was transferred onto a PVDF membrane at 300

mA for 75 minutes. Equal transfer was confirmed with Ponceau staining before blocking of the membrane for 1 hour in 5% nonfat milk with 0.01% TBS-T at room temperature. Primary antibodies (GPX4, TFR1, FTH1, GAPDH) were diluted at 1:1000 concentration in 5% milk, 1X tris-buffered saline and 0.01% tween were incubated on the membranes overnight at 4ºC. Following this incubation, secondary antibody at 1:10,000 concentration in 5% milk and 0.01% tween was incubated on the membrane for 1 hour at room temperature. The blot was washed with 5% milk,1X tris-buffered saline, 0.01% tween twice and then 1X Tris-buffered saline; 0.01% tween 2X before being probed with enhanced chemiluminescence (GE Healthcare) signal Blots were developed using the ProteinSimple Fluorchem R (R&D Systems) and analyzed using ImageJ software[80].

Cell Viability and Ferroptosis Induction

Cells were seeded at 4,000 cells per well in a 96 well plate and allowed to grow for 24 hours before treatment with 5 μM erastin, or a vehicle control (DMSO) along with 10 μg tetracycline for 24 hours. Co-treatment with 10 μM ferrostatin-1 (FER-1), a ferroptosis inhibitor, was used as a negative control. Cell viability was measured by PrestoBlue reagent (Thermo Fisher Scientific, Waltham, MA) by adding 10 μl of PrestoBlue reagent followed by incubation at 37 ºC for 20 minutes. Differences in fluorescence absorbance were measured using a Biotek Synergy HT plate reader. Differences in cell viability were determined by normalizing reductions in fluorescent absorbance relative to the vehicle control group for each cell line.

RNA Extraction

To assess gene expression changes following induction of ferroptosis, cells were seeded in a 6 well plate at 1 x 10^5 cell/well and incubated for 24 hours before treatment with 10 μM erastin and 10 μg tetracycline for 24 hours. After the 24-hour treatment, media was aspirated from the wells and cells were homogenized using 800 μl Trizol (Invitrogen Cat# 15596026) before being

collected into microcentrifuge tubes. Following a 5-minute incubation at room temperature, 160 µl of chloroform was added and the samples were mixed well and incubated for another 3 minutes at room temperature before being centrifuged at 12,000 x *g* for 15 minutes at 4ºC. The RNA containing supernatant was transferred to a fresh microcentrifuge tubes and 400 µl of isopropanol was added to each tube. RNA was precipitated at -80 ºC overnight. The next day, the samples were centrifuged at 12,000 x *g* for 15 minutes at 4ºC. The supernatant was poured off and the pellet was washed in RNase-free 75% ethanol, vortexed and centrifuged at 7500 x *g* for 5 minutes at 4ºC. Samples were then dried and re-suspended in nuclease free water. Concentration, OD 260/230 and OD260/280 ratios of RNA was measured using a Nanodrop (Thermo Fisher Scientific) spectrometer to assess RNA yield, and purity, respectively. RNA integrity was assessed by electrophoresis on a 1 % agarose/1% bleach gel at 100 V for 30 minutes. Samples were stored at -80ºC.

Real Time Quantitative Polymerase Chain Reaction

For cDNA synthesis, 1 µg total RNA, was DNase treated and then reverse transcribed using Superscript II (Thermo Fisher Scientific, Waltham, MA). Each sample was plated in duplicate with the addition of 1 µl of the PCR mastermix (SYBR green, primer and nuclease free water) to each well. The plate was then spun down at 1,000 x g for 5 minutes. Standard condition program was used with the ABI software with the 7900HT Fast Real-Time PCR Systems (Thermo Fisher Scientific, Waltham, MA). Cycles included one 2 min hold (50℃); one 10 min denaturation (95℃); 40 cycles of denaturation (95℃ for 15 sec) and annealing (60℃ for 1 min); and extension phase (95℃ for 15 sec followed by 60℃ for 15 sec followed by 95℃ for 15 sec). Genes were normalized to relative Peptidylprolyl Isomerase A (PIPIA) abundance using the $2^{-\Delta\Delta Ct}$ method. Primer sequences use for qPCR are listed in Table 1.

Table 1

Primer Name	Sense Primer	Antisense Primer	Accession No
PPIA	5'tgccatcgccaaggagtag	5'tgcacagacggtcactcaaa	NM_021130.5
TFRC	5'agttgaacaaagtggcacgagcag	5'agcagttggctgttgtacctctca	NM_001128148.1
SLC7A11	5'ctccgcgccggtgctttttg	5'ctccgcgccggtgctttttg	NM_014331.4
CISD1	5'ccttcacatccagaaagacaacc	5'ctcttcgttatgttttgtgtgagc	NM_018464.5
NOCA4	5'cagcagctctactcgttattgg	5'tctccaggcacacagagact	NM_001145260.1
ATG5	5'ctccgcgccggtgctttttg	5'cagattccgcgctccggtgg	NM_004849.4
DPP4	5'aaaggcacctgggaagtcatcg	5'cagctcacaactgaggcatgtc	NM_001935.4

Cyclo, cyclophilin; TfR1, Transferrin Receptor; SLC7A11, solute carrier family 7 member 11; CISD1, CDGSH Iron Sulfur Domain 1; NOCA4, Nuclear Receptor Coactivator 4; ATG5, autophagy related 5; DPP4, Dipeptidyl Peptidase 4

Aim 2: *To evaluate the impact of ferroptosis on IRP-dependent regulation of iron metabolism.*

Experimental Methods for Aim 2:

Electrophoretic Mobility Shift Assay (EMSA)

All cell lines described in Aim 1 were treated with 10 µM erastin and 10 µg tetracycline for 24 hours and harvested from 25T plates at 90% confluency. To harvest cytosolic protein, cells were collected and centrifuged at 1,000 x *g* for 5 minutes at 4ºC. The cell pellet was washed with 1X PBS and centrifuged at 1,000 x *g* for 5 minutes at 4ºC. Then, the cell pellet was re-suspended in 2-volumes cytosol buffer (1M HEPES 10mM, 10mM KCl, 0.1mM EGTA, 0.1 mM EDTA, 1M DTT, 0.1M PMSF, 100 X protease inhibitor cocktail) (Thermo Fisher Scientific, Waltham, MA). After 15 minutes, 10% v/v NP40 was added, and the samples were vortexed for 10 seconds then

centrifuged at 12,000 x g for 10 minutes at 4 C. Finally, the supernatant (cytosol) was removed to a fresh microfuge tube and stored at -80º C until use. Protein concentration was determined based on the bicinchoninic acid assay (Thermo Fisher Scientific, Waltham, MA, Cat #23209). Spontaneous IRP1 and IRP2 RNA binding activities was assessed by incubating 10 μg cytosolic protein with saturating levels of [^{32}P]-labeled RNA from the L-ferritin IRE as previously described[81]. Total IRP1 RNA binding activity was measured by adding 10 μg of cytosolic protein in the presence of 4% β-mercaptoethanol to saturating levels of RNA.

Assaying Lipid Peroxidation

To assay TP53-dependent differences in lipid peroxidation following erastin treatment, all cell lines were plated in an 8 well chamber slide (Ibidi, Martinsried, Germany) at 10,000 cells /well. Cells were treated 10 μM erastin and 10 μg tetracycline for 24 hours. The cells were then washed with 1X Hank's balanced salt solution (HBSS) before incubation with 5 μM BODIPY 581/591 C11and Hoechst stain (1:1000) for 10 minutes at 37ºC. Then, the mixture of 1X HBSS and reagent was aspirated and 1X HBSS was added to the cells. Cells were imaged using the BZ-X700 Life Science Microscope (Keyence). Increase lipid peroxidation was shown with oxidation of the polyunsaturated butadienyl portion of the dye resulting in a change from 590 nm to 510 nm excitation. Low photobleach settings and exposure times were held consistent through the imaging process. ImageJ software was used to measure differences in the ratio of red and green fluorescence intensities, which were then normalized to cell number by counting the number of Hoechst stained nuclei[80]. Increases in the ratio of green to red fluorescence intensities indicate an increase in lipid peroxidation.

Statistical Analysis:

One-way ANOVA was used for assessment of differences between cell lines and treatments. Differences will be statistically significant at 95% confidence (alpha=0.05). For post-hoc analysis

we used the Tukey LSD test. Student's t-test was used to compare cell type to their relative controls. All tests were performed using SPSS v23.0 software (IBM-SPSS; Chicago, IL, USA). All experiments will be repeated 3 times, n=3. All variables will include mean ± SEM.

CHAPTER IV

FINDINGS

TP53 mutation status influences sensitivity to erastin-induced ferroptotic cell death. Human lung carcinoma, H1299 cells, which are null for TP53, were co-transfected with a tetracycline repressor plasmid pcDNA6/TR, and a tetracycline inducible plasmid pcDNA5/TO containing a WT TP53 plasmid, or an exemplary contact (273H, 248Q,175H) or conformational TP53 mutant (282W, 245S, 249S). Tetracycline inducible WT and mutant TP53 protein expression was validated by western blot (**Figure 2)**. TP53 expression levels were variable between WT and TP53 mutation types. Induction of WT TP53 protein expression was lower than any of the TP53 mutants, and 273H, 248Q, and 175H mutant protein expression was higher than 282W, 245S, and 249S TP53 protein expression.

To assess the influence of distinct TP53 mutation types on ferroptosis sensitivity, we measured cell viability following treatment with erastin, a potent inducer of ferroptosis. TP53-dependent differences in ferroptosis sensitivity were determined by comparing differences in cell viability compared to H1299 cells expressing the WT TP53 plasmid. TP53 null H1299 cells and cells expressing the 282W mutation type did not exhibit differences in sensitivity to erastin treatment compared to the cells expressing WT TP53. However, induction of 273H, 248Q, 175H, 245S, and 249S TP53 mutations increased sensitivity to ferroptosis induction, as evidenced by

reduced cell viability compared to the WT TP53 expressing cells following 24 hours of erastin treatment (**Figure 3A**). Importantly, we also demonstrate that cell viability is decreased as a result of ferroptotic cell death as treatment with 10 μM ferrostatin, a potent ferroptosis inhibitor, was sufficient to rescue cells from ferroptosis (**Figure 3B**). These results indicate that induction of distinct TP53 mutation type is sufficient to increase sensitivity of cells to iron-mediated ferroptotic cell death.

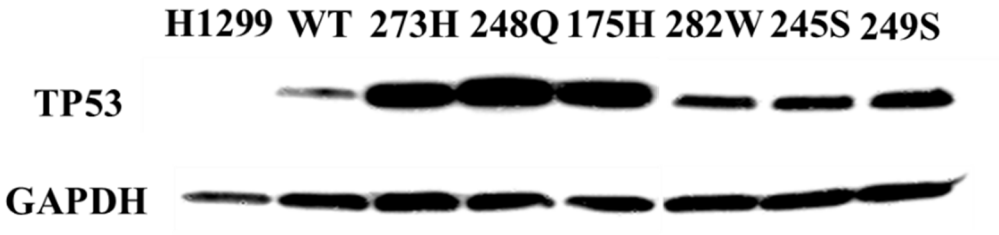

Figure 2 Validation of tetracycline inducible WT and mutant TP53 expression. H1299 (TP53 null) cells were transfected with either a tetracycline inducible wild-type (WT) TP53 or a representative contact (273H, 248Q, 282W) or conformational (175H, 245S, 249S) mutant TP53. Tetracyline inducible expression of WT and mutant TP53 expression was confirmed by western blot. GAPDH was used as a loading control.

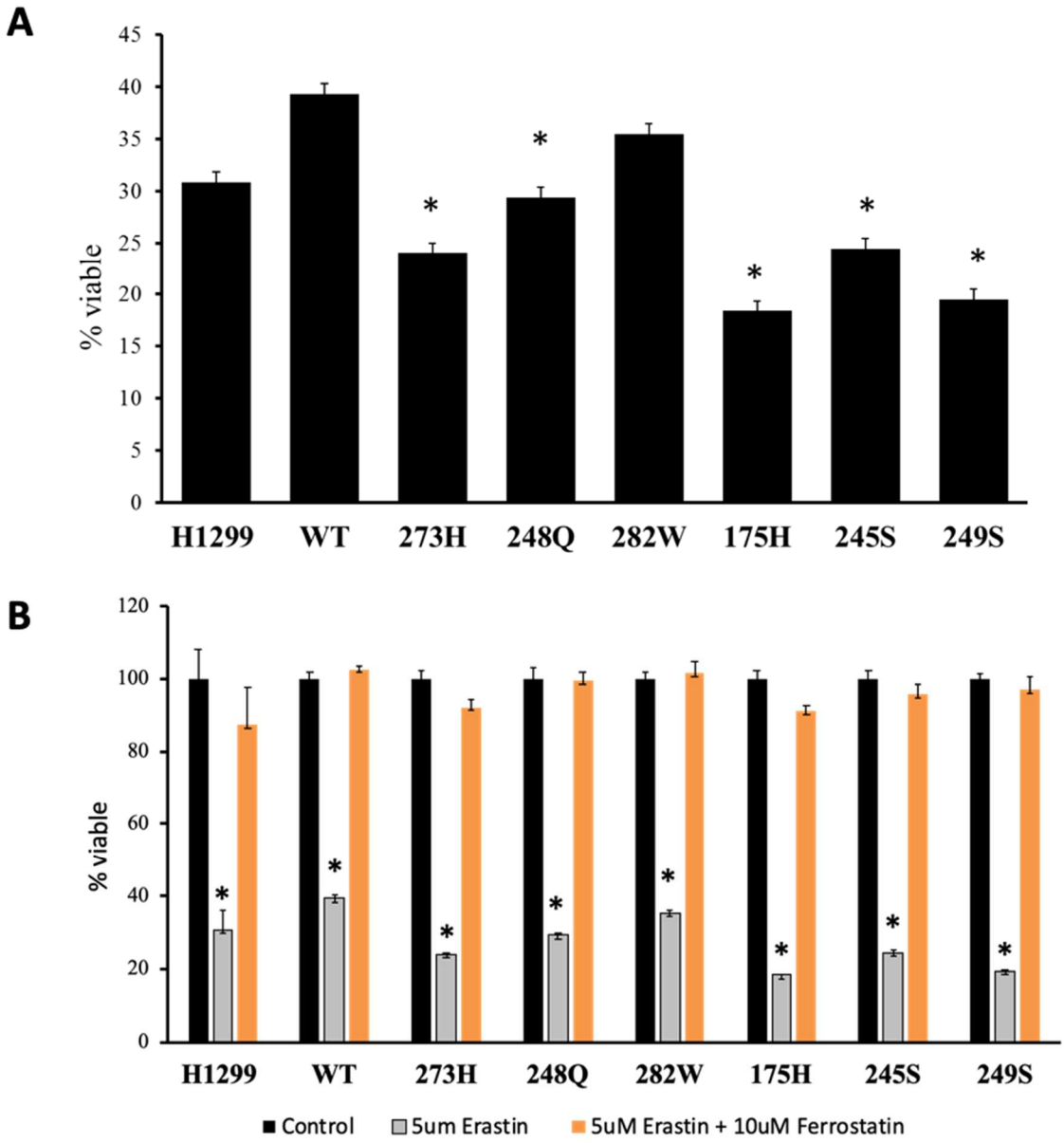

Figure 3 Influence of TP53 mutation status on ferroptosis sensitivity. Cell viability measured with PrestoBlue reagent following 24 hours of treatment with 5 μM erastin (**A**) A one-way between subjects ANOVA was conducted to compare the effects of TP53 mutation type on cell viability following erastin treatment (5 μM; 24 hours) in H1299, WT TP53, 273H, 248Q, 282W, 175H, 245S, and 249S cells. There was a significant effect of TP53 mutation type on cell viability after erastin treatment at the $p < 0.05$ level for the 8 cell types [$F(7, 16) = 16$, $p = 0.000$]. Post hoc comparisons using the Tukey LSD test indicated that WT P53 was significantly different from 273H (p=.001), 248Q (p=.014), 175H (p=.000), 245S (p=.001), and 249S (p=.000). *Denotes difference from WT TP53, $p<0.05$. (**B**) All cells were sufficiently rescued from ferroptosis by 10 μM ferrostatin, a potent ferroptosis inhibitor. *Denotes statistical differences from respective control, $p < 0.05$.

Ferroptosis induction differentially impacts IRP1/2 RNA binding activity in mutant TP53 expressing cells. To determine the impact of ferroptosis induction by erastin on IRP function and expression we quantitatively assessed spontaneous and total IRP RNA binding activity through an electrophoretic mobility assay. As iron-mediated lipid peroxidation is critical for ferroptosis induction we hypothesized that IRP RNA binding activity would be increased in response to erastin treatment. However, only cells expressing the 248Q (P=0.019), 175H (P=0.015), and 245S (P=0.027) TP53 mutation types exhibited statistically significant increases in spontaneous IRP RNA binding activity following erastin treatment (**Figure 4A and B**). Unlike IRP2, which is regulated at the level of protein degradation, IRP1 is regulated by the assembly of an Fe-S cluster which inhibits its RNA binding activity. The addition of β-mercaptaethanol prevents Fe-S assembly which allows for the measurement of total IRP1 protein levels[40,82]. This assay also informs us about the relative distribution of IRP1 in its RNA binding form or its Fe-S cluster containing enzymatic form[82]. Total IRP1 RNA binding activity was not affected by erastin treatment, indicating that changes in spontaneous IRP binding activity was not the result of changes in the total pool of IRP RNA binding capacity (**Figure 4A and C**).

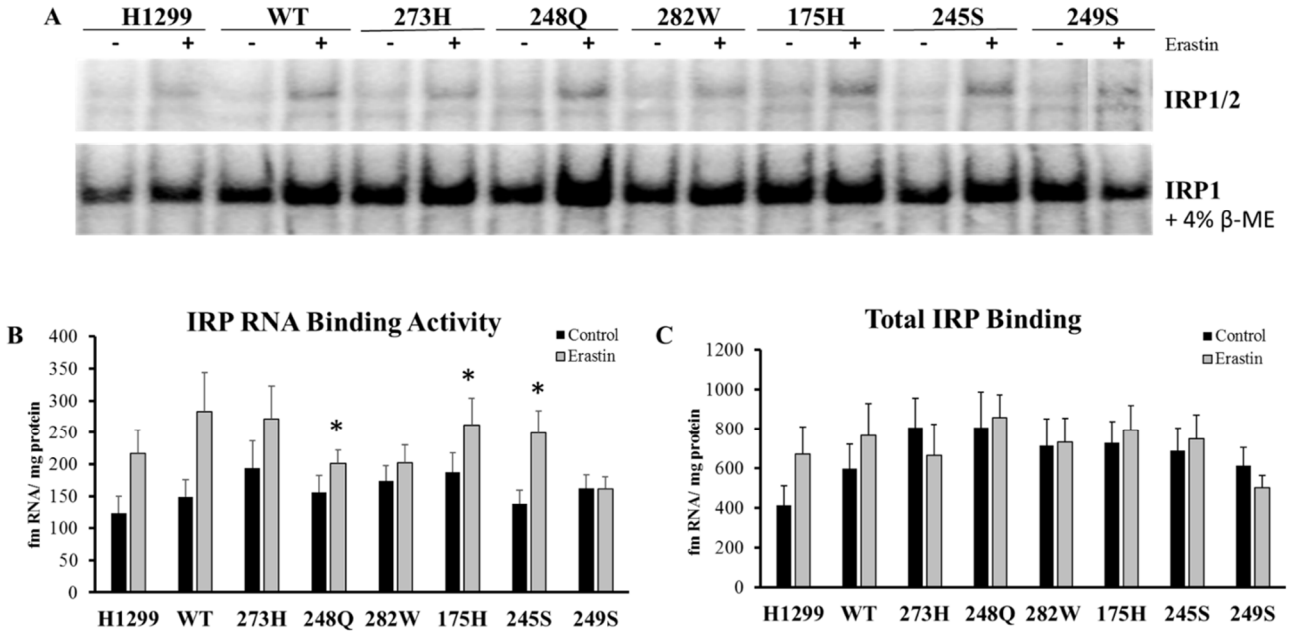

Figure 4 Effect of erastin treatment on IRP1/2 RNA binding activity in cells harboring WT TP53 or mutant TP53. Cells were treated with 10 μM erastin for 24 hours. (A) Spontaneous and total IRP RNA binding activity were assayed by EMSA *Denotes significant increase from cell types control, $p<0.05$. (B) Spontaneous IRP RNA binding activity was increased in cells expressing 248Q, 175H, and 245S TP53 mutants. (**C**) Total IRP RNA binding activity was not changed by erastin treatment.

TP53 mutation status influences lipid peroxidation levels increase following ferroptosis induction. To examine the impact of TP53-dependent differences in reactive oxygen species accumulation in response to ferroptosis induction we assessed lipid peroxidation following erastin treatment. There was a significant increase in lipid peroxidation following erastin treatment in all cells tested except for those expressing a 282W and 273H TP53 mutation (**Figure 5B**). To determine if differences in erastin responsiveness were due to TP53-dependent differences in basal lipid peroxidation, we compared differences in the level of oxidized probe in untreated cells expressing the distinct TP53 mutations types. While there was a significantly more basal lipid peroxidation in 273H mutant TP53 expressing cells, this was not true for the 282W mutants (**Figure 5C**).

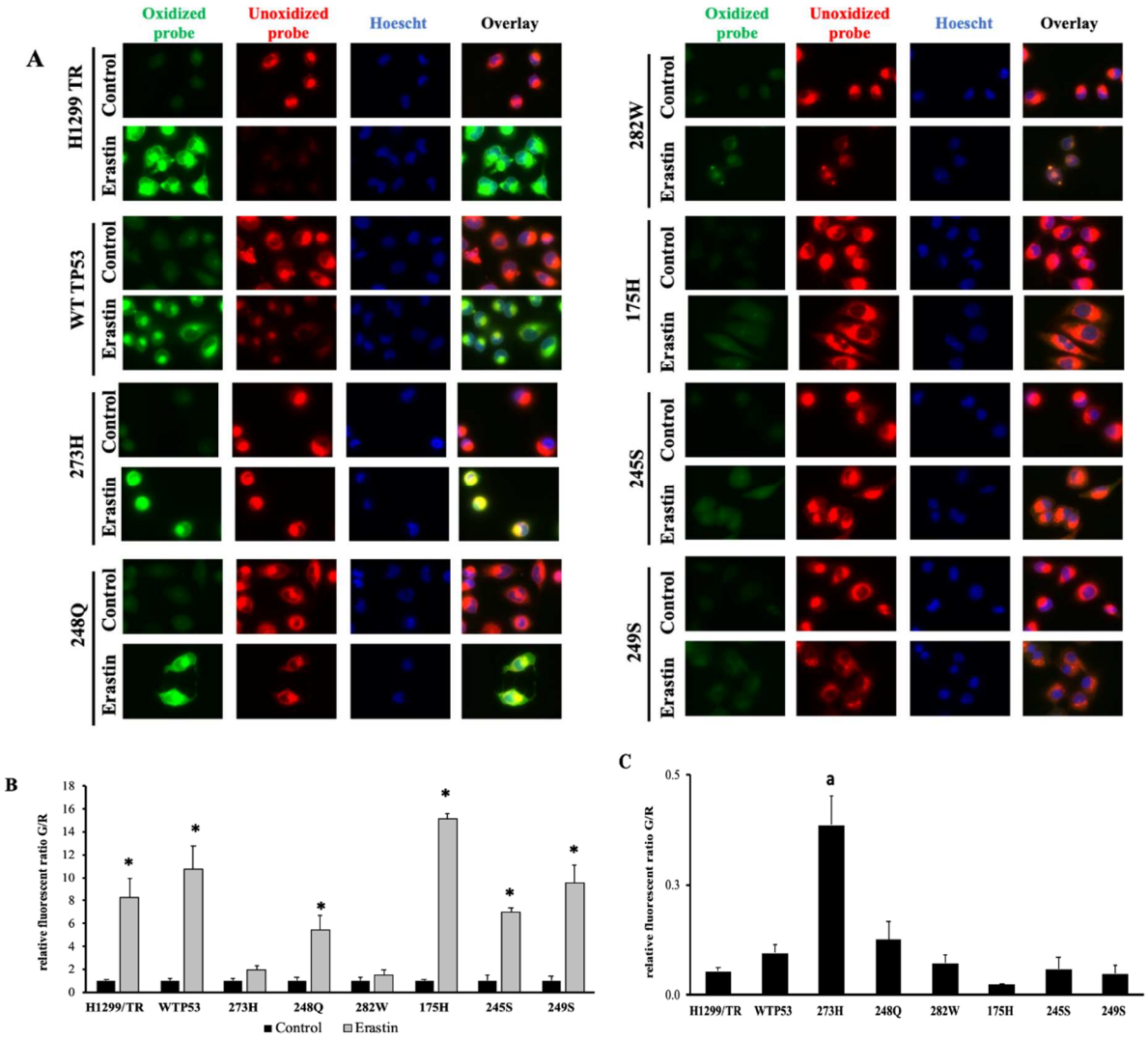

Figure 5 TP53-dependent differences in lipid peroxidation following ferroptosis induction. The same cells described in Figure 1 were treated with 10 uM erastin for 24 hours, after which cells were stained with BODIPY 581/591 Cll, a fluorescent lipid probe which shifts from red to green when oxidized. (**A**) Images that were quantified from BODIPY 581/591 C11 staining following normalization to Hoescht nuclear staining to account for differences in cell number. (**B**) A student's t-test was conducted to compare cell types to their control. * Denotes significant difference from control. (**C**) A one-way between subjects ANOVA was conducted to compare the effects of TP53 mutation type on lipid peroxidation in H1299, WT TP53, 273H, 248Q, 282W, 175H, 245S, and 249S cells. There was a significant effect of TP53 mutation type on basal oxidized probe at the p <0.05 level for the 8 cell types [F (7,16) = 13.563, p= 0.000]. Post hoc comparisons using the Tukey LSD test indicated that WT TP53 was significantly different from 273H (p= 0.000). An **a** denotes significance from WT TP53.

Ferroptosis induction and TP53 status do not influence iron related gene expression. To examine the influence of TP53 mutation types and ferroptosis induction on iron related gene expression, we assessed relative mRNA expression of TFRC, NCOA4, ATG5, and CISD1 from total RNA using real time quantitative PCR analysis. There was not a significant TP53-dependent change in NCOA4, ATG5, or CISD mRNA expression (**Figure 6**). There was also no significant changes following erastin treatment in any of the iron-related mRNAs examined. However, there was a significant increase in SLC7A11 mRNA expression in WT, 273H, 248Q, 282W, 175H, 245S, and 249S following erastin treatment (**Figure 6E**). SLC7A11 plays a role in the transport and regulation of cellular antioxidant response by regulation of glutathione and cystine transport, and is upregulated following erastin treatment[14,75]. Thus, it serves as a positive control indicating that ferroptosis was induced despite the lack of lack of change in iron-related gene expression.

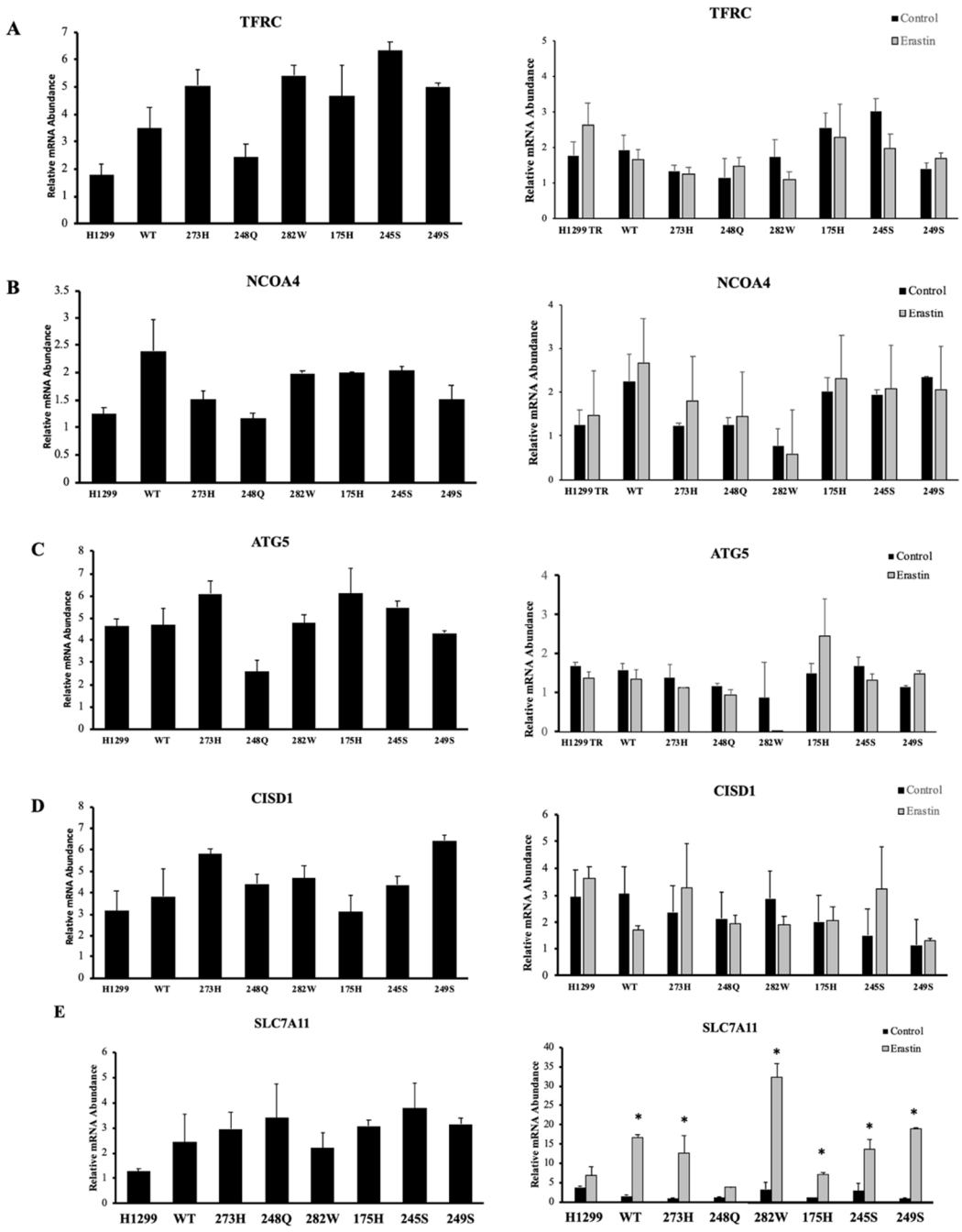

Figure 6 Induction of distinct TP53 mutations types does not impact iron related gene expression following following ferroptosis induction. (A) Relative mRNA expression of TFRC (B) NCOA4, (C) ATG5, and (D) CISD1. A one-way between subjects ANOVA was conducted to compare the effects of TP53 mutation type on lipid peroxidation in H1299, WT TP53, R273H, R248Q, R282W, R175H, G245S, and R249S cells. There was a significant effect of TP53 mutation type on TFRC gene expression at the $p < 0.05$ level for the 8 cell types [$F(7,16) = 5.302$, $p = 0.003$]. Post hoc comparisons using the Tukey LSD test indicated that H1299 was significantly different from 282W ($p = 0.027$) and 245S ($p = 0.004$).

Levels of antioxidant and iron related protein levels are differentially altered by erastin treatment in cells expressing distinct TP53 mutation types. To examine the influence of erastin treatment on protein levels of ferritin, transferrin receptor and glutathione peroxidase 4 (GPX4) we used western blot to assess relative protein levels following ferroptosis induction in each of the cell lines described above. Consistent with our qPCR findings, there was no significant changes in transferrin receptor protein expression following erastin treatment in any of the cell lines examined (**Figure 7A**). There was, however, a significant increase in ferritin expression following ferroptosis induction in 282W ($p=0.004$), and 249S ($p=0.013$) TP53 mutant expressing cells, but a significant decrease in ferritin expression in the 273H mutants ($p=0.020$). (**Figure 7B**). Expression of the potent antioxidant, glutathione peroxidase 4, significant decreased following erastin treatment only in cells expressing the 273H TP53 mutation (**Figure 7C**).

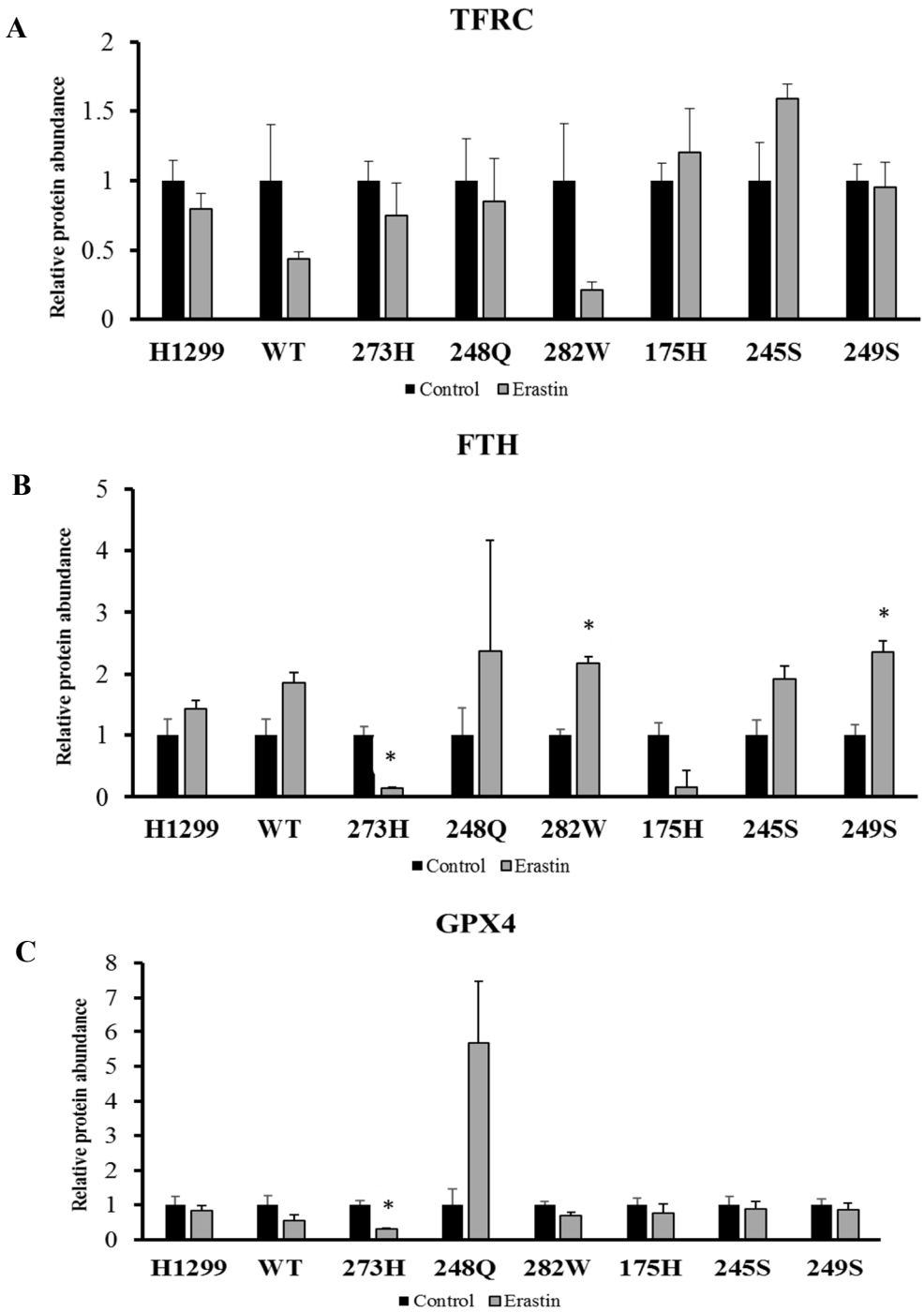

Figure 7 Relative protein abundance following TP53 induction and ferroptosis induction. Western blots of TFRC, FTH, and GPX4 protein expression in TP53 null H1299 cells or following induction of TP53 (WT, 273H, 248Q, 282W, 175H, 245S, and 249S) and treated with 10 uM erastin for 24 hours. Relative expression was quantified to anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as a loading control. Student's t-test was used to compare to relative control. *Denotes statistical significance to respective control, $p < 0.05$.

CHAPTER V

DISCUSSION

Wild-type TP53 has been implicated as an important mediator of ferroptosis sensitivity in human cancer cells, though contradictory roles have been reported[14,79,83,84]. These mixed findings are likely attributable to the context-dependent TP53-mediated upregulation of CDKN1A expression, which appears to be critical for suppression of ferroptosis[14]. Moreover, posttranslational modifications and/or genetic mutations within TP53 can render it unable to induce CDKN1A in some cell types resulting in increased ferroptosis sensitivity[83,85]. However, the mutants in these studies were acetylation defective mutants and not representative of the more common TP53 mutations within the DNA binding domain most often observed in human cancers. In this study we utilized isogenic cell lines expressing the six most common TP53 mutation types in human cancers to examine the impact of TP53 mutation status on sensitivity to ferroptosis. We established that induction of distinct TP53 mutations alone significantly increased sensitivity to ferroptotic cell death.

Though hundreds of TP53 mutation types have been identified, the majority occur in the DNA binding domain and are categorized as either contact or conformational. Contact mutations are missense changes in the amino acids of the protein that come into contact with the DNA binding domain thereby impairing the transcriptional activity of the protein without effecting

protein structure. Conformational mutations on the other hand both interfere with normal DNA binding and alter the proteins overall structure[16]. The distinction between these two TP53 mutation categories is functionally important because mutation type significantly influences mutant TP53 binding partners[19,86-88]. Of the three contact mutation types investigated in the current study, only the 273H and 248Q TP53 mutant expressing cell lines were more sensitive to ferroptotic cell death than WT TP53 expressing cells; whereas the 282W mutants responded similarly to the WT TP53 expressing cells. On the other hand, the three conformation mutation types examined in this study (175H, 245S, and 249S) were all consistently more sensitive to ferroptosis induction than WT TP53 expressing cells. Further investigation is needed to determine whether conformational type TP53 mutants are uniformly more susceptible to iron-mediated cell death.

We have previously established that induction of distinct TP53 mutation types differentially impacts IRP RNA binding activity and diminishes IRP responsiveness to changes in cellular iron availability[89]. As such, we hypothesized that mutant TP53-dependent differences in ferroptosis sensitivity might be influenced by mutant TP53-dependent differences in IRP RNA binding activity in response to erastin treatment. Indeed, IRP RNA binding activity was significantly increased following ferroptosis induction in the 248Q, 175H, and 245S TP53 mutants. However, IRP RNA binding activity in the 273H, 282W and 249S expressing mutants was unchanged by ferroptosis induction. Thus, erastin-mediated increases in IRP RNA binding activity cannot fully explain the increased sensitivity to ferroptotic cell death in all mutant TP53 expressing cell types.

To investigate if the increase in IRP RNA binding activity was indeed responsible for the increase in ferroptotic sensitivity in the 248Q, 175H, and 245S expressing mutants, we examined the expression of IRP target genes, TFRC and ferritin. We hypothesized that increased IRP RNA binding activity in these cells would result in increased TFRC expression and decreased ferritin

expression, thereby promoting ferroptotic cell death by increasing free iron availability. However, we did not observe any changes in TFRC mRNA or protein expression following erastin treatment in any of the cell lines examined. Similarly, ferritin expression was not changed in any of the cell lines for which an increase in IRP RNA binding activity was observed. Further confounding our initial hypotheses, ferritin expression was decreased in the 273H expressing mutants, but increased in the 249S and 282W mutants. Though these findings are inconsistent with our hypothesis, they are in agreement with our previous studies indicating that ferritin may be regulated independently of IRP in mutant TP53 expressing cells[89].

The degradation of ferritin via ferritinophagy is an IRP-independent mode of ferritin regulation with an established role in ferroptotic cell death[90,91]. Therefore, we investigated the potential for ferritinophagy to contribute to ferritin regulation and ferroptosis sensitivity in mutant TP53 expressing cells. We did not detect differences in the expression of any of the ferritinophagy related genes following erastin treatment in any of the cell lines examined. Thus, the mechanisms contributing to increased ferroptotic sensitivity in mutant TP53 expressing cells are complex and may not be consistent between distinct TP53 mutation types. Further investigation is warranted however because increased ferritin expression in the 282W mutant TP53 expressing cells, which were less sensitive to erastin treatment, may be indicative of a unique protective mechanism against ferroptosis in this particular TP53 mutation type.

Independently of how iron becomes available, it is the peroxidation of lipids by free iron within that ultimately leads to ferroptotic cell death. Therefore, we investigated if mutant TP53-dependent changes in reactive oxygen species accumulation in response to ferroptosis induction could explain the observed differences ferroptosis sensitivity. DAPI staining was used to control for differences in cell viability following erastin treatment, so that changes in fluorescent intensity ratios could be normalized to total cell number. Intriguingly, we found that only 5 of the 6 mutant TP53 expressing cell lines had significant increases in lipid peroxidation. This was a

somewhat surprising finding as each of these cell lines were susceptible to ferroptotic cell death, albeit to varying degrees.

To further investigate why the 273H and 282W TP53 mutant expressing cell lines did not undergo increased in peroxidation in response to erastin treatment we analyzed basal levels of lipid peroxidation following induction of TP53 expression alone. The 273H TP53 expressing mutants displayed significantly higher levels of basal lipid peroxidation than any of the other cell lines investigated, which could explain the lack of an additional increase in lipid peroxidation following erastin treatment. Contrarily, despite a lack of erastin responsiveness, induction of 282W mutant TP53 expression did not influence baseline levels of lipid peroxidation either. Intriguingly, the 282W mutant was the only TP53 mutant that was not more sensitive to erastin treatment than WT TP53 expressing cells. These findings indicate that 282W mutant may have more compacity to combat lipid peroxidation than other TP53 mutation types through a yet undefined mechanism. Future studies should examine differences in antioxidative capacity between cells expressing distinct mutant TP53 types.

Under normal cellular conditions, System x_c^-, which is composed of SLC3A2 and SLC7A11, imports cystine to produce glutathione, a necessary cofactor for GPX4 elimination of lipid peroxides[76-78,92,93]. In this study, we have examined the impact of ferroptosis induction by treating cells with erastin, a potent, System x_c^- inhibitor[78,92,94]. Consistent with previous reports, we also observed a significant increase in SLC7A11 mRNA expression following erastin treatment in each of the cell lines tested, with the exception of the 248Q TP53 mutants[75]. Intriguingly, the induction of SLC7A11 expression following erastin treatment was quite variable between the mutant TP53 expressing cell lines. The 282W TP53 expressing mutants displayed the greatest increase in SLC7A11 expression among the TP53 mutants examined and were also the least sensitive to erastin-mediated ferroptosis induction. Thus, it is tempting to speculate that cells

expressing the 282W TP53 mutation type have an enhanced compensatory response to System x_c^- inhibition.

To date, the targeting of mutant TP53 has primarily focused on restoring its wild-type activity, or promoting its degradation, while iron chelation has been a primary emphasis for the development of iron-based chemotherapy[72-74]. In this study we have established that induction of certain TP53 mutation types increases sensitivity to ferroptotic cell death. These findings are novel because they describe an approach that would allow for the exploitation of mutant TP53 expression to more favorably induce iron-mediated cell death via the activation of ferroptosis. We have also demonstrated that the IRP response to erastin treatment is dependent upon TP53 mutation type and is not essential for ferroptosis induction. Future studies should investigate the IRP-independent modes of iron regulation in mutant TP53 expressing cells.

CHAPTER V

CONCLUSION

Iron is an essential and a potentially toxic nutrient that can contribute to both the initiation and progression of cancer[1]. The tumor suppressor TP53 protects against carcinogenesis by contributing to the regulation of cellular iron homeostasis[95]. Unfortunately, however, TP53 is the mutated in nearly half of all human cancers. Mutations in TP53 can lead to both loss of tumor suppressive functions and the acquisition of oncogenic traits, but the influence on cellular iron homeostasis has yet to be fully described.

Ferroptosis is an iron-dependent mode of nonapoptotic cell death with vast chemotherapeutic potential[12,78,96,97]. Driven by the iron-dependent accumulation of lipid reactive oxygen species (ROS), the import of iron by TFRC-mediated iron uptake is an essential component of ferroptotic cell death[98]. The increased expression of IRP2 and degradation of FTH1 have also been touted as critical contributors to ferroptosis induction, presumably as a means of increasing the redox-active labile iron pool[78,91]. Yet, the contribution of the IRE-IRP system to iron accumulation during ferroptotic cell death has not been investigated until now.

Our lab has previously demonstrated that expression of distinct TP53 mutation types impairs IRP responsiveness to changes in cellular iron availability[89]. Thus, we hypothesized that disruptions in IRP-mediated signaling pathways would contribute to increased ferroptotic

sensitivity in mutant TP53 expressing cells. We did observe increased in IRP RNA binding activity in 248Q, 175H, and 245S mutants compared to WT TP53 expressing cells following ferroptosis induction. However, increased IRP RNA binding activity alone cannot fully explain the increased sensitivity to ferroptotic cell death in these cell types as the predicted corresponding changes in TFRC and ferritin expression were not detected. Moreover, IRP RNA binding activity was not increased in 273H and 249S mutants, but these cell types also exhibited increased sensitivity to ferroptosis induction.

Intriguingly, transferrin-bound iron uptake, via the IRP target TFRC, is essential for ferroptosis[98] . Though no changes in TFRC expression were observed in the present study, the question remains as to why TFRC would continue to import iron following ferroptosis induction at the cost of cell death. Research investigating the regulation of TFRC upon ferroptosis induction has produced conflicting results. Wang *et al.* reported reduced TFRC expression following erastin treatment[99]. Such results are consistent with an appropriate cellular response, wherein IRPs sense a relative cellular iron overload and decrease mRNA binding to reduce TFRC expression and subsequently cellular iron uptake. Conversely, however, Alvarez *et al.* reported an increase in TFRC expression following erastin treatment[100]. The authors speculated that the increase in TFRC expression resulted from a decrease in Fe-S biogenesis/stability and a subsequent increase in IRP1 RNA binding activity. Nonetheless, neither IRP1 nor IRP2 expression or activity were assessed in either of these studies. The inconsistency between observations of TFRC responsiveness between these studies, and ours, is likely due to the vast differences in experimental models. Nonetheless, our findings have enhanced the current knowledge of the field

by demonstrating that iron availability during ferroptosis can be mediated via IRP-independent mechanisms.

The most interesting of our findings in the present study were the TP53-dependent differences in response to ferroptosis. TP53, "the guardian of the genome", is responsible for regulation of energy metabolism, autophagy, angiogenesis, and iron homeostasis[9,10,89]. TP53 increases ferritin expression and promotes iron storage decreasing the availability of iron to become reactive[91]. Wild-type TP53 has also been previously reported to be protective against ferroptotic cell death[14]. However, TP53 is highly subject to genetic mutations, and when TP53 in mutated there is not only a loss of wild-type function but there is an acquisition of oncogenic properties[16,19,86]. No matter the mutation types, TP53 wild-type functions are impaired[19]. In this study, we have demonstrated that ferroptosis induction is a viable approach for the chemotherapeutic targeting of tumor cells expressing distinct TP53 types. Our findings are strengthened examining the most prevalent TP53 mutations that represent exemplary models of both contact and conformational mutants.

As these mutation types represent some of the most prevalent TP53 mutations in human tumors, these findings are relevant to a variety of clinically important cancers[16].

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VITA

Laurie Renee Thompson

Candidate for the Degree of

Master of Science

Thesis: IMPACT OF FERROPTOSIS INDUCTION ON IRON METABOLISM IN CANCER CELLS HARBORING TP53 MUTATIONS HERE IN ALL CAPS

Major Field: Nutritional Sciences

Biographical:

Education:

Completed the requirements for the Master of Science in Nutritional Sciences at Oklahoma State University, Stillwater, Oklahoma in December, 2020.

Obtained Bachelor of Science in Nutritional Sciences at Oklahoma State University, Stillwater, OK/United States in 2018.

Experience:
Graduate Research Assistant
Graduate Teaching Assistant

Professional Memberships:
Oklahoma Academy of Nutrition and Dietetics
Academy of Nutrition and Dietetics