ABSTRACT

The P53 transcription factor, encoded by the human gene TP53, is the most commonly mutated gene in human cancers. The P53 transcription factor suppresses tumor growth through the regulation of genes with diverse biological functions. Once the TP53 gene is mutated, P53 proteins not only lose their tumor suppression abilities, but also contribute to tumor growth. One way mutant P53 can contribute to tumor growth is to alter the regulation of iron-sulfur (Fe-S) cluster-containing proteins, thereby increasing iron uptake and availability. Understanding such alterations in function is important because Fe-S cluster proteins play a role in both energy metabolism and DNA repair enzymes, both of which can suppress the progression and expansion of cancerous tumors. This thesis work examines how TP53 mutation status influences Fe-S cluster protein regulation, and subsequently iron homeostasis in tumor cells. First, we assessed the activity of the Fe-S containing enzymes using both cytosolic and mitochondrial aconitase, in cell lines expressing a variety of TP53 mutations. Specifically, we used human cancer cell lines that express the six most commonly observed TP53 mutations, these six represent nearly 25% of all TP53 mutations in human cancers. We found that TP53 mutations significantly influence how the activity within Fe-S cluster enzymes is regulated. Thus, our ongoing studies are focused on investigating the mechanisms by which TP53 mutation status influences the regulation of genes involved with Fe-S cluster biogenesis and consequently, Fe-S cluster containing protein activity. This research is important because it will help determine how homeostatic control of iron metabolism is maintained in cancer cells expressing distinct TP53 mutations. Such findings could have significant implications for further facilitating the development of metabolic interventions and iron-targeted chemotherapy strategies.

INTRODUCTION

Normal Functions of P53:

TP53 gene plays a significant role in tumor suppression. The TP53 gene encodes the P53 protein. Under normal conditions, wild-type P53 functions as a sequence-specific transcription factor that helps regulate the expression of proteins that aid the cell during stress. For example, in response to DNA damage, the binding of P53 to its transcriptional targets can inhibit cell cycle progression, promote senescence, or induce apoptotic cell death [1]. The TP53 protein also regulates the transcription of genes involved in angiogenesis, senescence, autophagy, and metabolism [2]. Because of the proteins' abilities to preserve cellular integrity and repair DNA damage, some researchers have referred to P53 as the ‘guardian of the genome’ [3].

How P53 Regulates Iron:

One mechanism P53 functions as a tumor suppressor is by promoting the sequestration of iron into its storage form, which reduces its availability for cellular proliferation and participation in free radical formation [4]. Specifically, P53 transcriptionally regulates the expression of the iron-sulfur cluster assembly enzyme (ISCU). Following activation by P53, Fe-S cluster biogenesis is enhanced [2]. The Fe-S clusters are then integrated into proteins which help with the citric acid cycle, oxidative phosphorylation, and genomic stability, and reduce the availability of redox active iron [2].

Conversely, cells that lack P53, or express mutant P53, tend to accumulate iron in response to DNA damage. This is a possible explanation for why disruptions in Fe-S cluster biogenesis can cause genome instability [3]. For example, increased ISCU expression has been shown to protect cells from iron overload acting as a mediator of P53 to balance iron levels within the cell [3].

Indeed, decreased ISCU expression in human liver cancer tissues is associated with P53 mutations and cancer progression [5].

Mutations in the P53 Gene:

More than half of all human cancers have been found to have a mutation of the P53 transcription factor, thus making P53 the most commonly mutated gene in human cancers [6]. The majority of TP53 mutations are missense mutations, single base-pair substitutions, within the DNA-binding domain that interfere with its ability to transcriptionally regulate genes necessary for repressing tumor development [5]. However, some TP53 mutations don't just result in the loss of tumor suppressive functions but can also contribute to enhanced tumor growth. Thus, it is important to distinguish both TP53 mutation subtypes and phenotypes.

Iron Serves as a Target for Cancer Therapy:

Cancer cells require high amounts of iron to sustain proliferative capacity, because of this dependence, it stands to reason that the same nutrient could also act as an effective strategy to combat tumor development. However, our lab has previously shown that distinguishing between TP53 mutation types is also important in this regard because cells expressing distinct TP53 mutation types differentially respond to changes in iron availability [7]. One way to target iron depletion is to regulate iron handling through the use of an iron chelator, but iron chelators restrict both tumor and systemic iron availability which can negatively impact patient outcomes. Another way to target the excess iron in cancer cells is through the induction of ferroptosis, a form of iron-mediated programmed cell death, but ferroptosis sensitivity is also dependent upon TP53 status [8, 9].

The goal of this project is to examine how TP53 mutation status influences Fe-S cluster protein regulation, and subsequently iron homeostasis in tumor cells. The importance of these studies is underscored by the fact that iron is an essential, yet potentially toxic nutrient to both tumor cells and healthy cells. Thus, understanding how to manipulate iron availability in specific cell types, while minimizing the impact on patient iron homeostasis are essential components to the development of iron-targeted chemotherapeutic strategies.

METHODS

Cell Culture

Cells containing endogenous WT TP53 (SW48) or an endogenous R273H (MDA-MB-468), R248Q (HCC70), R282W (NCI-H510), R175H (AU565), G245S (SU.86.86), or R249S (BT549) TP53 mutation were obtained from the American Type Culture collection (ATCC). This panel of human cancer cell lines was selected because these mutations represent the six most commonly observed TP53 mutation types. The panel represented a range of cancer types and locations throughout the body (**Table 1**). For example, the AU565 cell line is derived from a patient with breast carcinoma. Cells were maintained in RPMI or DMEM (Corning) supplemented with 10% FBS or 100 µg/ml penicillin-streptomycin solution, per provider instructions. The cells were stored in an incubator set to 37° C with 95% humidity and 5% CO_2. Cells were cultured at a density of 1x10^5 cells/ml in-6-well plates and either left untreated (controls) or treated with 100 µM desferoxamine (DFO) for 24 hours. DFO is an iron chelator; therefore, when cells are treated with DFO they are supposed to become iron deficient.

Table 1 Human Cell Line Panel.

TP53 Mutation Type	Cell Line	Location in body
WT TP53	SW48	Colon
R273H	MDA-MB-468	Mammary gland
R248Q	HCC70	Duct, Mammary gland
R282W	NCI-H510	Lung
R175H	AU565	Breast, Mammary gland
G245S	SU.86.86	Pancreas
R249S	BT549	Breast, Mammary gland

Aconitase Activity Assay

To assess how TP53 mutation status influences the activity of Fe-S cluster containing proteins, we measured both cytosolic and mitochondrial aconitase activity under standard and iron-deficient conditions. Mitochondrial aconitase is an enzyme involved in the citric acid cycle, and cytosolic aconitase is the enzymatic isoform of iron regulatory protein 1. The assay measures the conversion of citrate to isocitrate. This results in a colorimetric product (450 nm) that corresponds directly to the amount of enzymatic activity present.

First, we collected cells untreated (control), or treated with 100 μM DFO for 24 hours. Cell pellets were homogenized in 100 μL of ice-cold Assay Buffer (Sigma-Aldrich), and then centrifuged at 800 x *g* for 10 minutes at 4° C. This step removed any insoluble material. We then collected the supernatant as the cytosolic fraction.

The supernatant was then centrifuged at 20,000 x *g* for 15 minutes at 4° C to separate mitochondrial protein fraction. After the 15-minute spin, the pellet at the bottom of the tube was resuspended and sonicated in 0.1 mL cold Assay Buffer. The samples were then activated by adding 10 μL of Aconitase Activation Solution (Sigma-Aldrich) to 100 μL of sample and incubated on ice for an hour. After the samples were activated, 50 μL of each sample reaction mix was added to 2 wells in a 96-well plate. A sample blank was utilized to make sure all recorded measurements were within the range of the standard curve. Once all the samples had been loaded on the plate, they were left to incubate at 25° C for 30-60 minutes in the dark. The developer was added to each well after the incubation period ended and the samples underwent a second 10-minute incubation period. The plate was then placed in a plate reader (BioTek) and absorbance at 450 nm was measured. Aconitase activity was determined with the use of an

isocitrate standard reference solution and was normalized to the amount of total protein in a given sample.

Total RNA Isolation

To prepare our samples for total RNA extraction, media was aspirated from the 6-well plate and 0.8 mL of TRIzol Reagent (ThermoFisher) was added to each well to homogenize the cells. Each sample was then transferred to a labeled microcentrifuge tube. The homogenized samples then underwent a 5-minute incubation, which helped the nucleoprotein completely dissociate. Following the incubation period, 0.16 mL of chloroform was added, and all samples were vigorously shaken by hand for 15 seconds. The samples then underwent another 2-minute incubation period for at room temperature. Samples were then centrifuged at 12,000 x *g* for 15 minutes at 4° C. The mixture in the microcentrifuge tubes was then separated into three layers, a lower red phenol-chloroform phase, interphase containing DNA, and a colorless upper aqueous phase containing the RNA.

The aqueous supernatant containing the total RNA was transferred via micropipette to a new microcentrifuge tube for RNA precipitation. A precipitate was formed by adding 1 mL of 100% isopropanol to the aqueous layer. The samples were then vortexed and stored overnight at -80° C to precipitate the RNA. The RNA was collected by centrifugation at 12,000 x *g* for 30 minutes at 4° C. The supernatant was then removed, and the RNA pellets were washed with 1 mL of 75% ethanol. They were then centrifuged at 7,500 x g for 5 minutes at 4° C. The supernatant was removed, and RNA pellets were left to air-dry for 5 to 10 minutes. The pellets were then resuspended in DEPC-treated H_2O. All samples were then assessed for integrity and

concentration using agarose gel electrophoresis and NanoDrop 1000 (ThermoFisher), respectively.

Real-time Quantitative Polymerase Chain Reaction (qRT-PCR)

qRT-PCR was used to determine the differences in mRNA expression between the DFO-treated samples and the controls. First, we conducted a DNase treatment, and then 1 µg total RNA was reverse transcribed to cDNA using Superscript II (ThermoFisher). The relative abundance of transferrin receptor (TFRC), aconitase 2 (ACO2), iron-sulfur cluster assembly enzyme (ISCU), heme oxygenase 1 (HMOX1), and CDGSH iron sulfur domain 1 (CISD1) mRNA was then measured in duplicate by real-time qPCR using SYBR green chemistry on an ABI Fast 7900HT Real-Time PCR System (Applied Biosystems) and normalized relative to the abundance of Cyclophilin mRNA using the $2^{-\Delta\Delta Ct}$ method.

RESULTS

Both WT and mutant TP53 expressing cells are responsive to iron chelation and iron supplementation

To determine how TP53 mutation status influences the cellular responsiveness to changes in iron availability, cells expressing either WT TP53 (SW48) or an R175H TP53 mutation (AU565) were used. Cells expressing these two TP53 subtypes were used because our lab has previously shown that isogenic cell lines expressing exogenous WT and R175H TP53 displayed the most extreme iron-responsive phenotypes [12]. Cells were treated with either DFO or hemin. DFO is an iron chelator that acts by chelating iron pools and depletes intracellular storage of iron [10]. Hemin is a heme iron supplement that can be used to induce iron overload and oxidative stress [11]. Because DFO chelates iron, making cells iron deficient [10], we hypothesized that expression of the iron uptake protein, TFRC, would increase in response to DFO treatment decrease in response to hemin treatment. Indeed, both the WT and mutant TP53 expressing cells displayed modest reductions in TFRC protein expression in reponse to DFO treatment, and significantly reduced TFRC expression in response to the hemin treatment (**Figure 1**). These findings indicate that both WT and mutant TP53 expressing cells respond appropriately to changes in iron availability.

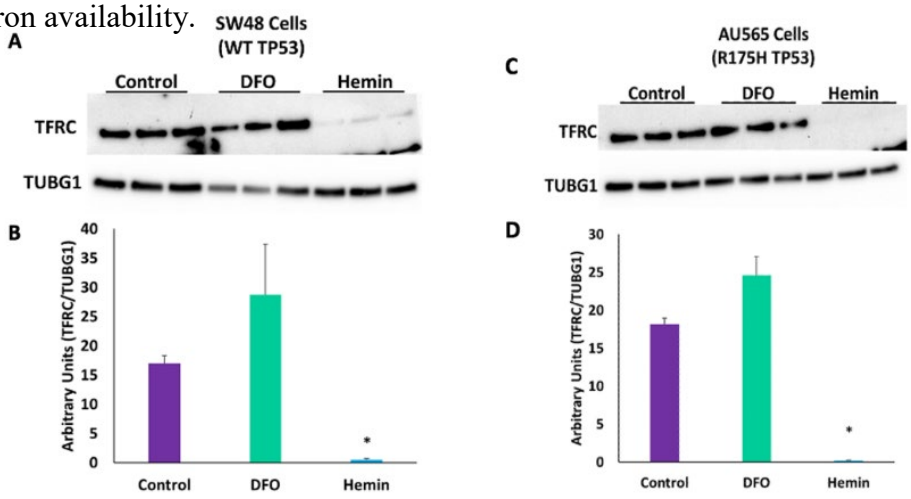

Figure 1 – Both WT and mutant TP53 expressing cells are sensitive to iron chelation and iron supplementation. TFRC protein expression was assessed by western blot in (A) WT TP53 expressing SW48 cells or (B) R175H TP53 mutant expressing AU565 cells following treatment with 50 μM DFO or 40 μM hemin for 24 hours (B) Relative expression levels were quantitated following normalization to anti-gamma tubulin (TUBG1) as the loading control. *Denotes statistical difference from respective controls, $p < 0.05$.

The enzymatic activity of Fe-S containing proteins is differentially impacted by iron availability in WT and mutant TP53 expressing cells

To assess the physiological impact of iron chelation on cells expressing distinct TP53 mutation subtypes, we measured the activity of two Fe-S containing isoenzymes with established roles in iron homeostasis, mitochondrial and cytosolic aconitase. Mitochondrial aconitase aids in energy production by converting citrate to isocitrate during the citric acid cycle. When the cell has low iron levels or increased oxidative stress, mitochondrial aconitase activity is inhibited. Cytosolic aconitase is a bifunctional protein that exists in an Fe-S cluster containing enzymatic form under iron replete conditions, whereas when iron is limiting, its Fe-S cluster is disassembled and it functions as the mRNA binding protein, iron regulatory protein 1 [13]. Because DFO treatment diminishes iron availability, and subsequently interferes with Fe-S assembly, we hypothesized that DFO treatment would decrease both mitochondrial and cytosolic aconitase activity. However, while cytosolic aconitase activity was repressed in cells expressing WT TP53 (SW48) and an R248Q TP53 mutation (HCC70), in most of the TP53 mutant cell lines, DFO treatment did not appropriately repress cytosolic or mitochondrial aconitase activity (**Figure 2**). The R175H mutant suprisingly showed an increase cytosolic aconitase activity in response to DFO treatment (**Figure 2A**).

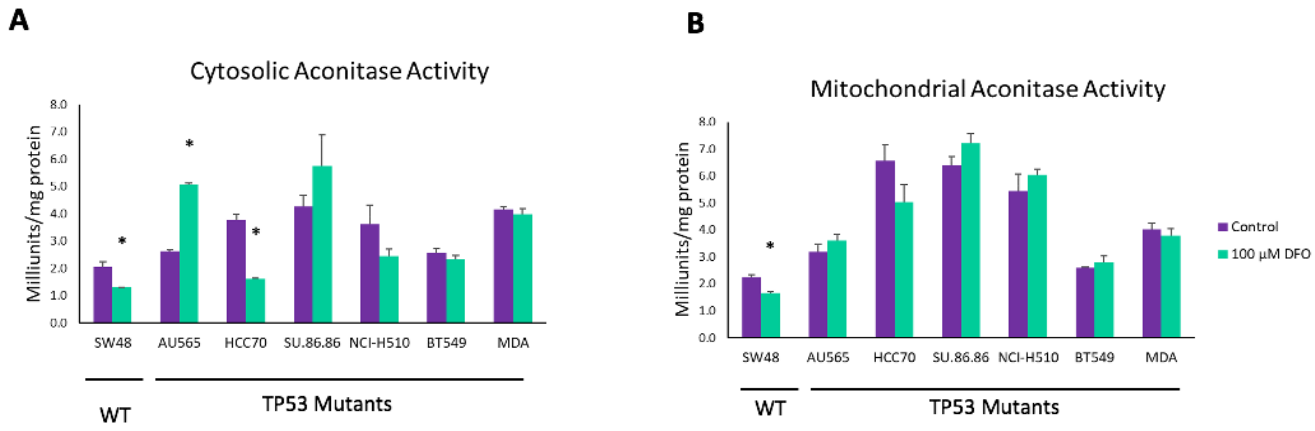

Figure 2. The influence of iron chelation on Fe-S cluster containing enzyme activity in WT and mutant TP53 expressing cells. Cells expressing endogenous WT TP53 (SW48) or an endogenous R175H (AU565), R248Q (HCC70), G245S (SU.86.86), R282W (NCI-H510), R249S (BT549), or R273H (MDA-MB-468) were treated with 100 µM DFO and untreated (control) for 24 hours. As expected, (**A**) cytosolic aconitase was significantly decreased in cells expressing WT TP53, and the cell line expressing the R248 TP53 mutation type. However, cytosolic aconitase activity in the R175H mutant TP53 expressing cell line increased in response to iron chelation. Similarly, (**B**) mitochondrial aconitase activity was reduced in cells expressing WT TP53 but was unaffected in any of the mutant TP53 expressing cell lines examined. *Denotes statistical difference from respective controls, $p < 0.05$.

DFO treatment does not alter Fe-S biogenesis related mRNA expression

To examine what might be contributing to the differences in the functional impacts of iron chelation in cells expressing distinct TP53 mutation types, we measured the relative mRNA expression of genes involved in mitochondrial iron transport (CISD), Fe-S cluster assembly (ISCU), and iron homeostasis (TFRC, ACO2, and HMOX1) following DFO treatment. We observed a significant increase in TFRC mRNA expression in the WT TP53 expressing SW48 cells and the R249S TP53 mutant expressing BT549 cell line, but in none of the other TP53 mutation types. These results indicate that, even with the longer treatment time, this lower DFO dose was not sufficient to elicit an iron deficient response in each of the cell lines tested. In support of this hypothesis, the expression of the other iron-related mRNAs we examined were all

changed in the SW48 cell line, but to a lesser extent in the BT549 cell line, and not at all in the AU565 or MDA cell lines. Intriguingly, expression of the mitochondrial iron importer, CISD1, and mitochondrial Fe-S cluster containing enzyme ACO2 were repressed in the R248Q TP53 mutant expressing HCC70 cell line. Thus, it is tempting to speculate that these cells may adapt to reduced iron availability by reducing mRNA iron utilization to preserve cellular iron homeostasis.

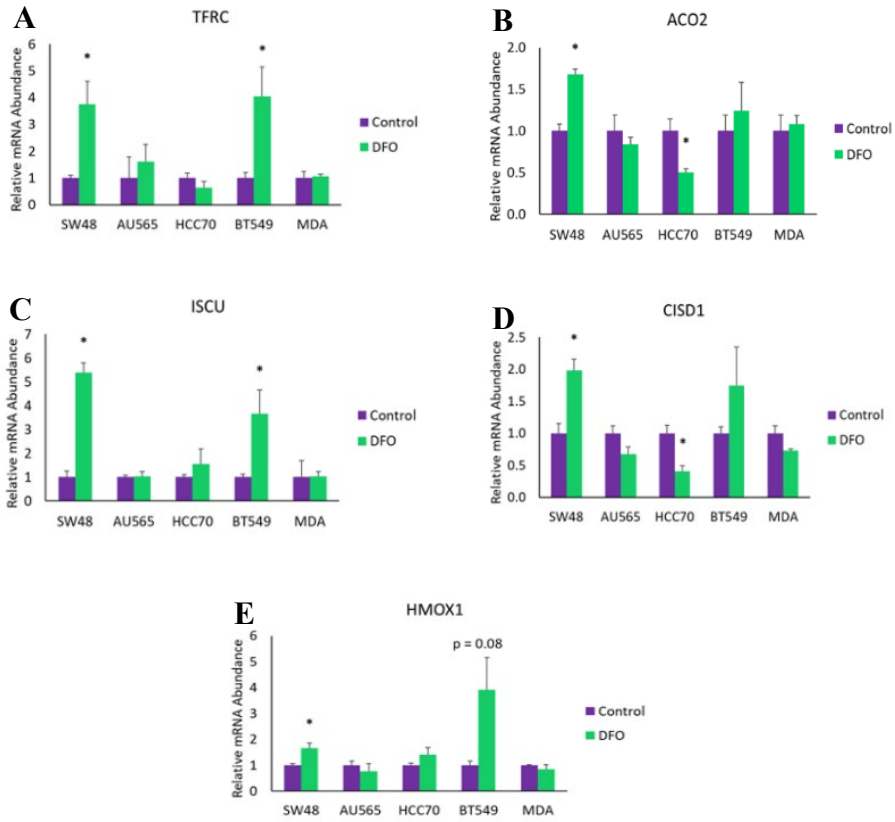

Figure 3. WT and mutant TP53 expressing cells exhibit different sensitivities to iron chelation. Cells expressing endogenous WT TP53 (SW48) or an endogenous R175H (AU565), R248Q (HCC70), R249S (BT549), or R273H (MDA-MB-468) TP53 mutation were left either untreated (Control) or treated with 50 μM deferoxamine (DFO) for 48 hours before isolation of total RNA. Relative mRNA expression of (**A**) transferrin receptor 1 (TFRC), (**B**) mitochondrial (ACO2), (**C**) iron-sulfur cluster assembly enzyme 1 (ISCU1), (**D**) CDGSH iron sulfur domain 1 (CISD1), and (**E**) heme oxygenase 1 (HMOX1) was assessed by qRT-PCR. *Denotes statistical difference from respective control, $p < 0.05$.

DISCUSSION

The tumor suppressor TP53 is the most commonly mutated gene in all human cancers, but in addition to loss of tumor suppressor functions, mutations in TP53 can also promote cancer progression by altering cellular iron acquisition and metabolism [4]. For example, cells that lack p53, or express a mutant p53, accumulate iron in response to DNA damage [14]. Mutations in p53 have also been shown to decrease tumor cell responsiveness to iron restriction [5]. Yet, inactivation of iron regulatory proteins can facilitate wild-type p53-mediated cell cycle arrest [4]. However, there is still much to learn about how distinct mutations in the TP53 gene alter protein regulation and iron homeostasis. As iron is necessary for cancer cell growth and proliferation, there is a critical need to understand how iron homeostasis is maintained in cells with mutated TP53. Our lab has previously shown that isogenic cell lines expressing exogenous TP53 mutants exhibit unique mutant TP53 subtype-dependent responses to iron restriction [7]. Further investigation revealed that these differences may be due to mutant TP53-dependent differences in Fe-S cluster biogenesis. The goal of this work was to confirm these findings in a panel of tumor cell lines expressing endogenous versions of these same mutant TP53 subtypes.

We hypothesized that iron chelation would decrease the expression of Fe-S biogenesis related mRNAs, leading to a decrease in protein activity. Although we saw a decrease in cytosolic and mitochondrial aconitase for WT TP53 and (SW48) and an R248Q TP53 mutation (HCC70), most of the endogenous mutant TP53 expressing cell lines did not appropriately repress enzymatic activity of Fe-S containing proteins. In fact, we observed an increase in cytosolic aconitase activity in cells expressing an R175H TP53 mutation. The changes, or lack thereof, in aconitase activity are likely explained by our observed lack of changes in iron- and Fe-S-related mRNA expression. Cells respond to iron deficiency by increasing the expression of

the iron importer, TFRC, to increase iron uptake. However, only WT TP53 expressing SW48 cells, and R249S mutant TP53 expressing cells significantly increased TFRC expression in response to our DFO treatment. These findings indicate that the dose and duration of DFO used in this study was not sufficient to induce iron deficiency. This treatment dose and timeframe was selected because we wanted to use the same treatment we had used to successfully elicit an iron deficient response in our exogenous mutant TP53 expressing isogenic cell lines. Nonetheless, these results confirm our initial findings demonstrating cells expressing mutant TP53 are less sensitive to reduced iron availability than cells expressing wild type TP53.

Currently, there are multiple iron-based cancer therapies being explored for use as cancer treatments. However, while iron is an essential nutrient, too much iron can eventually become toxic to both healthy and malignant cells. Thus, determining how to manipulate iron availability in mutant TP53 expressing tumor cells expressing is important. Our work highlights that TP53 mutation type also influences the response to iron, making it critically important to analyze the effect of distinct TP53 mutations on p53 function.

Future work should investigate the mechanisms contributing to mutant TP53-dependent responses to alterations in iron availability and determine how altering the dose and/or duration of treatment could improve outcomes. A better understanding of how these tumor cells modulate iron levels is key to finding ways in which we can establish an exploitable weakness for targeting mutant TP53 expressing cancers.

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