UNIVERSITY OF CALIFORNIA SAN DIEGO

Elucidating the Role of HSF1 and its Therapeutic Potential in Acute Myeloid Leukemia

A Thesis submitted in partial satisfaction of the requirements
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in

Bioengineering

by

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Professor Ludmil Alexandrov

2022

The Thesis of Yoon Joon (Dave) Kim is approved, and it is acceptable in quality and form for publication on microfilm and electronically.

University of California San Diego

2022

DEDICATION

To my father, Joo Woo Kim, my mother, Ji Yeon Oh, my sister, Chae Jeong Kim, and my girlfriend, Natalia Lopez Tome.

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VITA

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ABSTRACT OF THE THESIS

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The development of effective therapeutics to treat patients with acute myeloid leukemia (AML) has been challenged by the heterogeneous molecular landscape of the disease. One strategy to overcome the variability of therapeutic efficacy is to identify and target a universal cellular process upon which cancer cells are particularly dependent. Malignant growth increases the demand for protein biosynthesis, which can unbalance protein homeostasis (proteostasis) in cancer cells. Consequently, malignant cells can become addicted to stress response pathways that help restore proteostasis and support survival in the face of proteotoxic stress. Heat shock

factor 1 (HSF1) promotes various cancers by regulating proteostasis, metabolism, cell cycle, and multiple signaling pathways. However, the role of HSF1 in AML has not yet been thoroughly investigated and therapeutics targeting HSF1 have not been developed successfully. Here, we show that HSF1 inhibition exerts prominent anti-leukemic effects *in vitro* and *in vivo*. Genetic depletion of HSF1 in human AML cell lines significantly reduced cell growth and proliferation *in vitro*, slowed AML progression and extended survival *in vivo*, and dysregulated several oncogenic signaling pathways. In addition to its intrinsic anti-leukemic activity, HSF1 deletion sensitized AML cells to treatment with the proteasome inhibitor carfilzomib. Finally, we found that cysteamine, a type 2 transglutaminase inhibitor that restricts HSF1 activation, partially recapitulates the anti-leukemic effects of HSF1 ablation and produces supra-additive effects with carfilzomib. Together, our results indicate that human AML cells are highly dependent on HSF1 and propose HSF1 inhibition as an encouraging novel therapeutic avenue for treating AML.

INTRODUCTION

Acute myeloid leukemia (AML) is an aggressive hematologic malignancy characterized by the accumulation of immature hematopoietic cells with abnormal proliferation and differentiation. It has a poor prognosis marked by a dismal 30% 5-year survival rate and a high relapse rate[1]. Despite advances in medicine, the survival rate of AML patients has largely stagnated, highlighting a critical unmet need for the development of new AML therapies that produce complete remission and inhibit relapse[1].

Current AML Treatments and Obstacles

AML treatment typically consists of induction therapies that aim to produce complete remission and consolidation therapies that follow to manage and maintain remission. Standard induction therapy is the 7+3 regimen: 7 days of cytarabine and 3 days of daunorubicin. Since its approval by the U.S. Food and Drug Administration (FDA) in 1973, it has been the standard of care for most patients with AML. While the remission rate varies between 30% and 80% based on

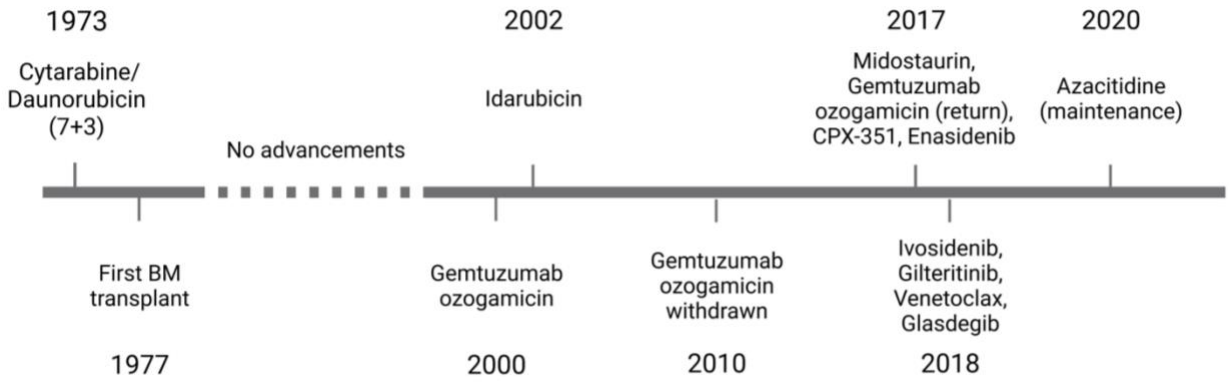


Figure 1. **History of FDA Approved AML Therapies.** Timeline of FDA approved clinical therapies for AML in the United States. Adapted from Carter et al., *Signal Transduction and Targeted Therapy* (2020).

the underlying factors of the patient, its long-term efficacy is poor and associated with frequent relapse[2]. Over the past several decades, numerous pre-clinical and clinical therapeutics have been developed to treat AML. However, the 7+3 regimen remains the first line treatment as novel AML therapies have failed to produce significant benefits over the standard combination for most patients.

One of the primary factors hindering the efficacy of AML therapies is the presence of leukemia stem cells (LSCs). LSCs are self-renewing leukemia cells that maintain and propagate

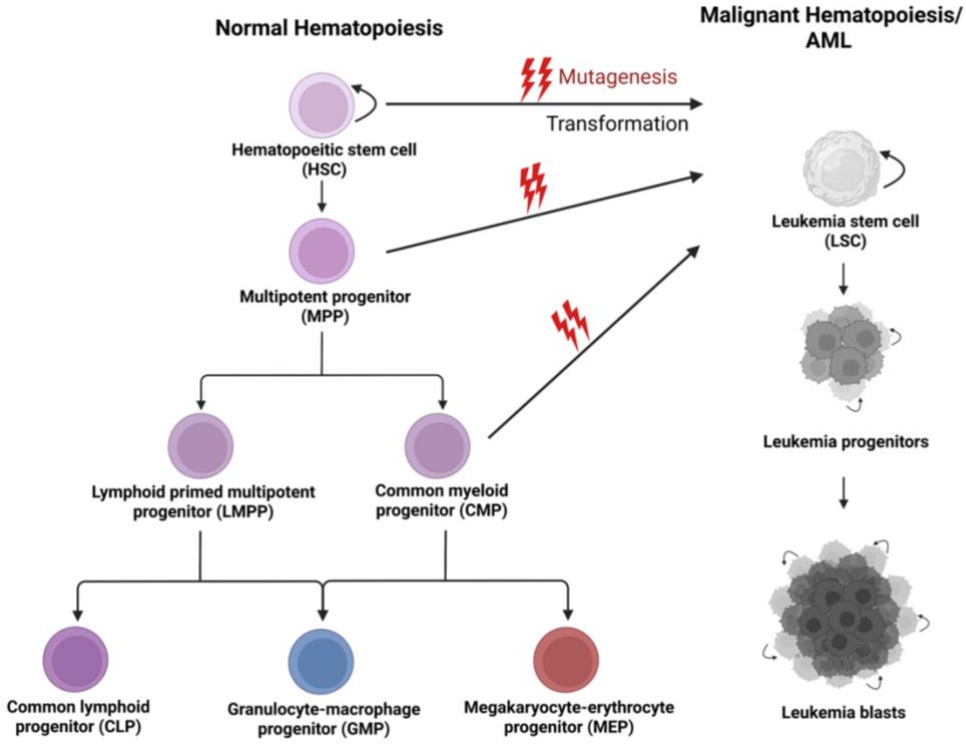


Figure 2. Hierarchy in Normal Hematopoiesis and Leukemic Progression.
In normal hematopoiesis, hematopoietic stem cells (HSCs) with self-renewal capacity give rise to multipotent progenitors (MPPs), which can differentiate into lymphoid primed multipotent progenitors (LMPPs) and common myeloid progenitors (CMPs). In turn, these can differentiate into common lymphoid progenitors (CLP), granulocyte-macrophage progenitors (GMP), and megakaryocyte-erythrocyte progenitors (MEP). From a series of mutations in the hematopoietic stem and progenitor cells (HSPCs), these cells can be transformed into leukemia stem cells (LSCs) with self-renewal and leukemia-initiating capacities. Leukemia develops and progress from the proliferation of LSCs and accumulation of leukemia progenitors and blasts. Adapted from Long et al., *Stem Cell Reviews and Reports* (2022)

malignant disease. LSCs are often resistant to conventional therapies, and their persistence leads to patients relapsing with advanced disease. LSCs arise from a series of mutations leading to differentiation arrest and malignant transformation of hematopoietic stem and progenitor cells (HSPCs)[3,4]. LSCs share several properties with HSCs, such as quiescence, self-renewal activity, and proliferative capacity[5,6]. Studies have also reported that LSCs overexpress drug resistant proteins such as BCL-2 and P-glycoprotein[7,8]. The combination of quiescence and enhanced drug resistance enables LSCs to evade AML therapy, and their self-renewal and proliferative capacity drives the onset of disease relapse. To eradicate LSCs, immunotherapy has emerged as a potential strategy. Studies have aimed to target LSC-enriched antigens such as CD33, CD123, and CD47[4]. These investigations produced the CD33-targeting antibody-drug conjugate gemtuzumab ozogamicin which showed clinical efficacy and obtained FDA approval for the treatment of CD33-positive AML patients. However, the clinical application of immunotherapies for LSCs has been limited in general because of the adverse effects on nonleukemic cells that express the targeted epitope and the inability to target a heterogeneous immunophenotypic landscape of LSCs.

Another critical obstacle against the efficacy of novel AML therapeutics is the genetic and molecular heterogeneity of the disease. The genetic landscape of AML is enormously diverse, and therapeutics have not been able to exert effectiveness in a broad range of patients. Under the World Health Organization classification, AML with mutations in NPM1, CEBPA, and RUNX1 are recognized as its entity in addition to BCR-ABL1 fusion gene, PML-RARA fusion gene, and 6 different chromosomal translocations[9]. Despite not having its classification, mutations in FLT3, IDH1/2, DNMT3A, and TET2 are also frequently observed in AML patients[2,3,10]. A strategy adopted to overcome the genomic complexity of AML is precision medicine. Currently, numerous targeted therapies are in clinical trials and in development to target specific vulnerabilities of AML

subtypes. For example, tyrosine kinase inhibitors and FLT3 inhibitors aim to reduce the pro-leukemic properties conferred by the constitutive activation of mutant FLT3[11]. Notably, midostaurin and gilteritinib have gained FDA approval for the treatment of relapse/refractory AML with FLT3 mutations[12,13]. Another example is IDH1 and IDH2 inhibitors, which mitigate the production of the oncometabolite 2-hydroxyglutarate (2-HG) from mutant IDH1/2 to eradicate AML cells with such mutations[14,15]. While efficacious on subsets of AML patients, many of the novel targeted therapies produce only modest effects, and treatments for AML patients with other mutations remain to be established[10]. Thus, there is a pressing need for the development of therapies that exploit a more universal cellular process to resolve the challenges stemming from expansive heterogeneity.

Protein Homeostasis

Protein homeostasis (proteostasis) governs the conformation, quantity, and location of proteins in a cell[16]. Proteostasis is regulated by a network of cellular processes that intricately coordinate protein synthesis, transport, folding, and degradation to maintain a balanced proteome[16,17]. Proteins are involved in innumerable roles in the cell, and their conformation substantially affects their functions. As a natural process, errors in transcription, translation, and modification can generate dysfunctional proteins[18,19]. Hence, proteins are commonly misfolded and damaged despite the folding process aided by numerous molecular chaperones.

There are several proteostasis pathways that cells employ to cope with the proteotoxic stress exerted by the accumulation of misfolded proteins. Misfolded proteins can be degraded by the ubiquitin proteasome system or autophagy. They can also be properly refolded into their functional conformation via molecular chaperones. Stress response pathways, such as the unfolded

protein response (UPR) and the heat shock response (HSR) can be activated to broadly reconfigure proteostasis network activity to help rebalance proteostasis. The consequence of proteostasis imbalance is well characterized in neurodegenerative diseases such as Parkinson's disease and Huntington's disease, but is less understood in malignant disease.

In cancer, increased protein synthesis is often required to sustain malignant growth and proliferation[20]. Elevated protein synthesis increases the biogenesis of misfolded proteins and confers proteotoxic stress on cells[21]. This raises the possibility that cancer cells may be unusually dependent on the proteostasis network and stress response pathways. However, the role of proteostasis regulation in AML and its potential as a therapeutic target have not been extensively investigated.

Proteasome is a Key Component of the Proteostasis Network and Cancer Therapeutic Target

Protein degradation via the ubiquitin proteasome system involves the tagging of degradation-bound proteins with ubiquitin. In this process, proteins are ubiquitinated through a three-enzyme cascade in which ubiquitin is activated by E1, transferred to and conjugated by E2, and ligated to its substrate by specific E3 ubiquitin ligases[22]. Polyubiquitinated proteins are deubiquitinated by the regulatory 19S subunit of the 26S proteasome, upon which the catalytic 20S subunit degrades the protein[22]. The 20S subunit is comprised of three active sites: caspase-like β1, trypsin-like β2, and chymotrypsin-like β5[23].

The proteasome can be suppressed to disrupt proteostasis by impeding 26S proteasome activity or deubiquitinating enzyme (DUB) activity[24]. Although DUBs such as USP15, PSMB5, and DUB3 promote survival and therapy resistance of malignant cells, DUB inhibitors have yet to demonstrate clinical efficacy[25–28]. On the other hand, several proteasome inhibitors have been

developed and approved for the treatment of hematologic malignancies. Bortezomib, a first-generation proteasome inhibitor that reversibly inhibits β5, has shown impressive efficacy in treating multiple myeloma as it improved overall survival, progression free survival, and response rate compared to the standard of care dexamethasone[29]. Since its FDA approval in 2003, it has been used for first line and relapse/refractory therapy for multiple myeloma and mantle-cell lymphoma[23]. Carfilzomib and ixazomib, second-generation proteasome inhibitors that irreversibly inhibit β5, then followed the success of bortezomib as they exhibited significant efficacy and obtained FDA approval in relapse/refractory multiple myeloma[30]. The efficacy of several other proteasome inhibitors such as oprozomib and marizomib are also being assessed in multiple myeloma[24]. Unfortunately, in AML, proteasome inhibitors have not shown significant efficacy as a stand-alone therapy or in combination with chemotherapy as most clinical trials failed to show significant improvement over the standard of care[31].

Autophagy is an Alternative Protein Degradation Pathway that Regulates Proteostasis

Another major protein degradation pathway that relieves proteotoxic stress is autophagy. Autophagy is typically activated in response to nutrient deprivation and stress and promotes survival by generating biosynthetic resources by degrading and recycling proteins and organelles. Autophagy consists of the initiation, nucleation and elongation of autophagosomes that engulf cellular material, followed by lysosomal fusion, and ultimately enzymatic degradation of the contents within autolysosomes[32]. Surprisingly, autophagy has a conflicting role in cancer initiation and cancer survival. Autophagy can obstruct cancer initiation by preventing oncogenic processes such as inflammation and genomic instability[33]. On the other hand, autophagy can enhance cell

survival and often confers therapy resistance in advanced stages of cancer by supporting the increased metabolic and proteostatic load[32,33].

Despite these dichotomous effects, therapeutic approaches are primarily designed to inhibit autophagy. Inhibition of molecules involved in the initiation step such as ULK1 and ATG4B have shown promising results in suppressing cancer growth and progression, but their efficacy has been limited to preclinical models[34,35]. The most successful autophagy inhibitors have targeted the lysosome. In addition to the preclinical efficacy observed from several lysosomal inhibitors, chloroquine significantly enhances the potency of chemotherapy in glioblastoma and has shown promise in clinical settings[36,37]. However, there is increasing evidence that the anticancer effect of chloroquine may be independent of autophagy inhibition[32,38,39]. Its analog hydroxychloroquine has also been adopted as a combination for glioblastoma, but it did not produce a significant improvement over the standard of care[40]. In AML, the addition of an autophagy inhibitor increases the cytotoxicity of cytarabine and enhances its therapeutic effect in preclinical settings[41,42]. The only clinical trial involving an autophagy inhibitor in AML (NCT 02631252) was terminated due to the inability to accrue, and the clinical value of autophagy inhibitors for AML has not yet been determined[43].

The Unfolded Protein Response is Activated in Response to Proteostatic Stress

In addition to protein degradation systems, cells employ adaptive stress response pathways to restore proteostasis. The endoplasmic reticulum (ER) UPR is one of these pathways. It is comprised of three UPR sensors - PERK, IRE1α, and ATF6a - which trigger unique downstream signaling cascades[44]. In response to protein stress, these UPR sensors are released from BiP/GRP78 which normally sequesters these sensor proteins in the ER. The UPR initially

attenuates translation through the phosphorylation of eIF2α by PERK to decrease protein synthesis, reduce the proteostatic load, and enhance the protein folding capacity in the ER[44]. Moreover, phosphorylated eIF2α induces the expression of ATF4. As a transcription factor, ATF4 promotes the transcription of its target genes that can augment protein folding, redox homeostasis, autophagy, amino acid metabolism, and apoptosis[45]. The cascade mediated by IRE1α also functions to relieve proteostatic stress. Upon stress induction, IRE1α cleaves XBP1 which encodes the active transcription factor XBP1s. XBP1s induces the expression of its target genes that rebalances proteostasis through protein folding, secretion, and ER-associated degradation (ERAD)[46]. Under stress conditions, ATF6 is cleaved and produces ATF6f. The activity of ATF6f enhances ERAD to relieve proteostatic load and restore proteostasis[44].

When protein stress exceeds the proteostasis buffering capacity and cannot be resolved, the UPR can promote cell death. Chronic UPR activation can induce the ATF4-mediated transcription of CHOP, which promotes apoptosis[47]. At the same time, the UPR directly participates in apoptosis by activating BH3-only proteins and pro-apoptotic proteins of the BCL-2 family as well as downregulating anti-apoptotic proteins[44,45,48]. Because of its ability to reduce stress and induce apoptosis, UPR has both oncogenic and tumor suppressive activity. For example, PERK supports tumor growth by promoting redox homeostasis and metastasis through a cascade mediated by CREB3L1[49,50]. Furthermore, PERK mediates terminal UPR and apoptosis crucial for the therapeutic efficacy of proteasome inhibitors in multiple myeloma[51]. On the other hand, PERK also produces the anti-leukemic effect of an experimental compound GSK621 by repressing metabolism and priming apoptosis[52]. Similarly, RNA degradation via regulated IRE1α dependent decay can restrict cancer cell migration and angiogenesis in glioblastoma[53]. In contrast, inhibition of IRE1α endonuclease activity can be used therapeutically as subsequent XBP1 splicing promotes

cell death and impairs tumor growth in myeloma[54]. Efforts to therapeutically target the UPR in cancer should focus on driving the activation of a terminal UPR response.

The Heat Shock Response is Activated in Response to Proteotoxic Stress

The heat shock response (HSR) is another adaptive stress response pathway that restores proteostasis. The HSR is characterized by the induction of a wide array of heat shock proteins (HSPs) which primarily function as molecular chaperones that mediate protein folding. The HSR and HSP activity are largely driven by the master transcription factor Heat shock factor 1 (HSF1). At steady state, HSF1 typically exists as an inactive monomer sequestered in the cytoplasm by chaperones such as HSP90 and HSP70[55]. Under stress conditions, chaperones bind to unfolded or misfolded proteins and release HSF1. HSF1 is thus activated and undergoes trimerization, nuclear translocation, and phosphorylation. HSF1 subsequently binds to and promotes the transcription of numerous HSPs, as well as other proteostasis and cell survival factors, enabling cells to cope with stress and rebalance proteostasis.

In addition to their roles in protein folding, these chaperones are involved in various cellular processes that can support cancer development. Within the HSP70 family, HSP72, HSPA6, and HSC70 contribute to malignancy by enhancing autophagy, proliferation, or by suppressing cell death[56,57]. HSP90 family proteins support malignancy by engaging in the processing of notable oncoproteins and signaling pathways such as AKT in the PI3K/AKT pathway, IL-6 in the JAK/STAT pathway, and BCR-ABL upstream of ERK/MAPK pathway[58,59]. As potential therapeutic targets in cancer, inhibitors of HSP90 have shown the most noticeable progress. HSP90 inhibitors such as 17-AAG, IPI-504, BIIB021, BIIB028, and PF-0429113 advanced to phase I/II clinical trials as therapies for melanoma, gastrointestinal stromal tumors,

refractory solid tumors, and hematologic malignancies[60–64]. While well tolerated overall, HSP90 inhibitors require further investigation as they did not produce clinically significant improvements, with some compounds exhibiting adverse toxicity[60–64]. Upregulation of other chaperones, such as HSP72, was observed with HSP90 inhibition, indicating the presence of a compensatory cross talk between different components of the HSR[62,65]. Silencing HSF1 prevents the compensatory HSR activation and enhances the effect of HSP90 inhibitors in hepatocellular carcinoma[66]. Thus, rather than targeting a single HSP, inhibiting HSF1 is a promising strategy that can bypass compensatory activity between various chaperones while maintaining tumor suppressive effects of prospective HSP inhibitors.

Non-canonical Functions of HSF1 Also Support Cancer Growth and Progression

Although primarily known for its role in the HSR, HSF1 has various roles outside of proteostasis maintenance. HSF1 regulates a distinct pathway in cancer cells that supports malignancy not only through protein folding but also via cell signaling, cell cycle regulation, metabolism, immune response, and adhesion[67]. For its various pro-survival functions, HSF1 is overexpressed and its upregulation is correlated with poor prognosis in various types of cancer, including breast cancer, ovarian cancer, renal cell carcinoma, and lung adenocarcinoma[68]. Several pre-clinical studies have demonstrated that inhibiting HSF1 is a promising target for cancer therapeutics as it reduces its diverse pro-survival functions. In melanoma cells, reducing HSF1 activity through MEK inhibition increases amyloidogenesis and sensitivity to proteasome inhibitor treatment, illustrating the proteostatic contribution of HSF1 to malignancy[69]. On the other hand, in pancreatic cancer, HSF1 prevents cell death via the mitochondrial anti-apoptotic protein SMAC and its genetic silencing decreases proliferation[70]. Furthermore, HSF1 supports glycolysis, a key

metabolic pathway utilized by cancer cells by activating LDH-A[71]. Loss of HSF1 reduced LDH-A activity and impaired the growth of breast cancer and hepatocellular carcinoma cells[71,72]. In the blood, inhibition of HSF1 impedes the progression of T-ALL through metabolic defects resulting from the downregulation of mTORC1 and MAPK/ERK signaling[73]. In FLT3-mutant AML, translation inhibition via rohinitib treatment inactivates HSF1 and decreases glucose metabolism and growth while increasing sensitivity to FLT3 inhibitor treatment in FLT3-mutant AML[74,75]. Despite the ability to target several universal cellular processes to which malignant cells are vulnerable, research on the role of HSF1 in AML is severely lacking and the potential of HSF1 as an AML therapeutic target remains to be elucidated.

Here, we examine the effect of HSF1 knockout in fully transformed human AML cells to understand the role of HSF1 in AML growth and progression. Using an HSF1 deficient AML cell line generated using the CRISPR-Cas9 system, we found that HSF1 depletion reduces AML growth and proliferation both *in vitro* and *in vivo*. Surprisingly, the loss of HSF1 did not induce significant proteotoxic stress in AML although we observed significant changes in the expression of proteostasis related genes. In addition, loss of HSF1 appeared to disrupt the oncogenic ERK/MAPK signaling pathway and tumor suppressive PTEN signaling pathway. However, HSF1 depletion sensitized AML cells to drugs that disrupt proteostasis. Treatment with the proteasome inhibitor carfilzomib resulted in increased apoptotic and anti-proliferative effects in HSF1-deficient AML cells. Together, our findings demonstrate a key role for HSF1 in promoting AML growth and progression, and highlight the potential of HSF1 as a therapeutic target to disrupt multiple pathways in AML.

RESULTS

HSF1 is broadly expressed by human AML cell lines and activated in response to stress

HSF1 has a critical role in maintaining cellular fitness by regulating proteostasis, but the extent to which it is expressed and activated in AML is not fully established. We examined HSF1 expression in four human AML cell lines TF1a, MV4-11, OCI-AML2, and OCI-AML3. TF-1a cells have TP53 and NRAS mutations, MV4-11 cells harbor an MLL-AF4 fusion and FLT3-ITD, OCI-AML2 cells carry an MLL-AF6 fusion and DNMT3A mutation, and OCI-AML3 cells have mutations in DNMT3A, NRAS, and NPM1. We chose this combination of cell lines to reflect some of the mutational heterogeneity of AML and to determine if approaches to disrupting proteostasis could be broadly applicable across different AML subtypes and mutations. HSF1 was expressed in all four human AML cell lines (Figure 3).

Next, we tested whether HSF1 was activated at steady state in AML cells and further activated in response to stress. Phosphorylation at Ser326 is essential for HSF1 activation and serves as a biomarker for activated HSF1[69,76]. HSF1 was modestly activated in human AML cell lines (Figure 3). However, heat shocking the cells at 42°C substantially increased HSF1 expression and dramatically increased p-HSF1 (Ser326) across all 4 human AML cell lines (Figure 3). These data indicate that HSF1 is broadly expressed and activated in fully transformed human AML cells irrespective of the heterogeneous mutational landscape.

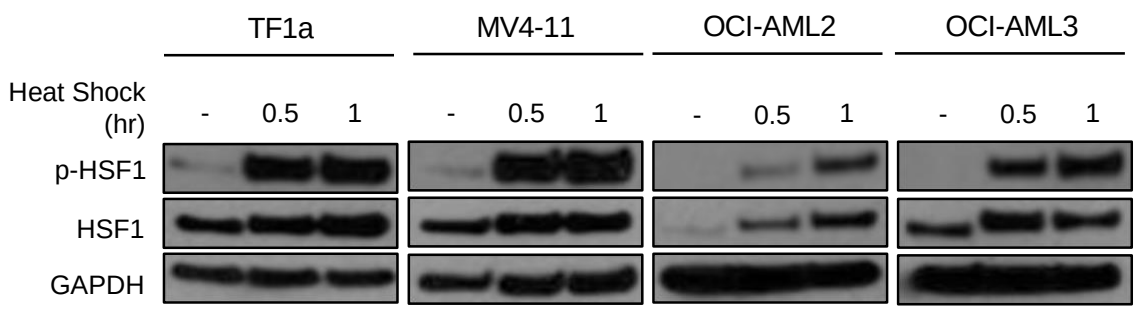


Figure 3. HSF1 is broadly expressed by human AML cell lines and activated in response to stress.

Western blot analysis of HSF1 and p-HSF1 (Ser326) was performed from the human AML cell lines TF1a, MV4-11, OCI-AML2, and OCI-AML3. Cells were incubated at 42°C for 0, 0.5, or 1hr prior to collection.

HSF1 deficiency reduces AML growth and proliferation *in vitro*

To examine the functional impact of HSF1 in AML, we generated HSF1 knockout (KO) AML cell lines using CRISPR-Cas9. Three HSF1 KO cell lines were generated by transfecting TF1a cells with a plasmid containing gRNA-Cas9 (Figure 4A). We confirmed the loss of HSF1 by Western blot. Both steady state expression and stress-induced activation were lost in all three KO clones, validating the generation of HSF1 KO TF1a cells (Figure 4B).

Next, we tested the impact of HSF1 loss on AML cell growth, apoptosis, and proliferation. HSF1 KO TF1a cells exhibited reduced growth as compared to parental TF1a cells (Figure 4C). HSF1 deletion did not significantly increase AML cell apoptosis (Figure 4D), but it significantly reduced proliferation (Figure 4E). These data indicate that HSF1 enhances AML cell growth by supporting increased proliferation.

Figure 4. HSF1 deficiency reduces AML growth and proliferation *in vitro*

(A) Schematic figure of CRISPR-Cas9 mediated HSF1 KO in TF1a cells.

(B) Western blot examining HSF1 expression and activation in parental (WT) and HSF1 deficient (KO) TF1a cells. Cells were incubated at 37°C or 42°C for 1 hour prior to analysis.

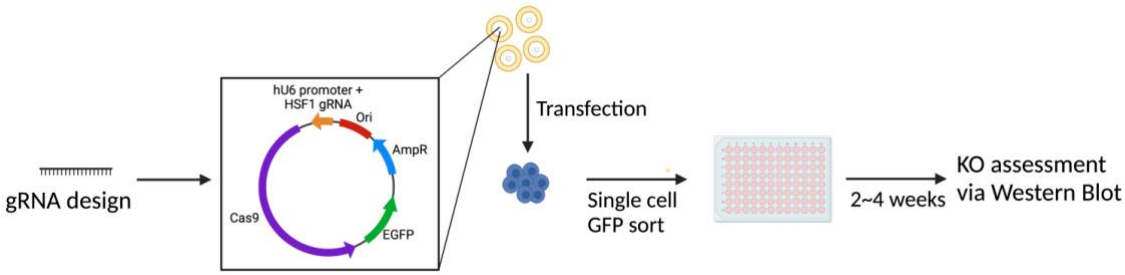
(C) Growth curve of WT and HSF1 KO TF1a cells. Cells were plated in triplicates in 6 well plates at $2x10^5$ cells/ml (n=4). Viable cells were counted based on trypan-blue dye exclusion every 24 hours after plating.

(D) Frequency of Annexin V+ in parental (WT) and HSF1 KO TF1a cells. Cells were plated in duplicates at 10^6 cells/ml in 96 well plates (n=3).

(E) Frequency of EdU+ dividing in parental (WT) and HSF1 KO TF1a cells. Cells were plated in duplicates at 10^6 cells/ml in 96 well plates (n=3).

(C-E) Data represent mean ± SEM. Significance was assessed using a t test (n.s: not significant, *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$).

A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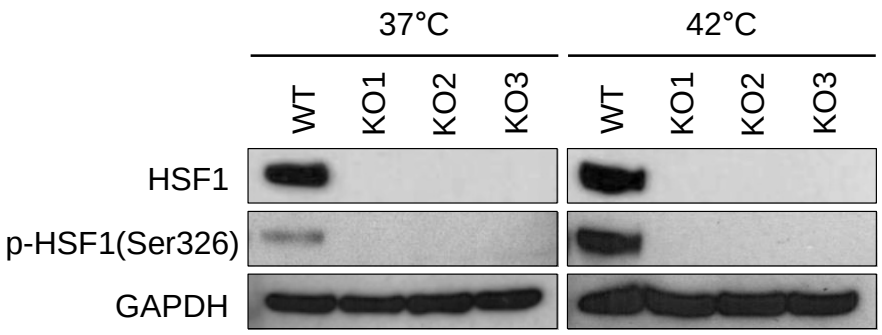
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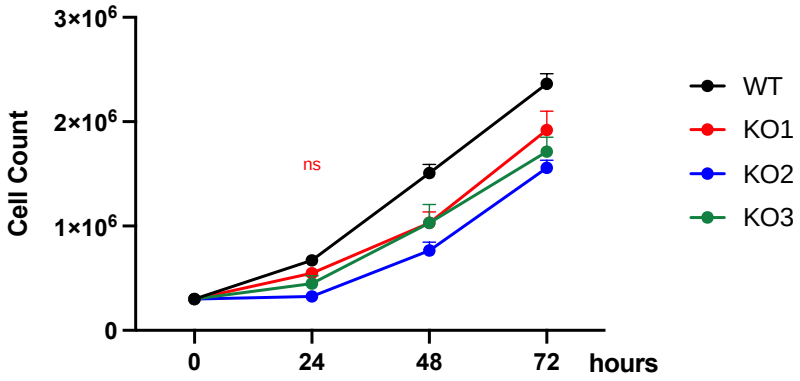
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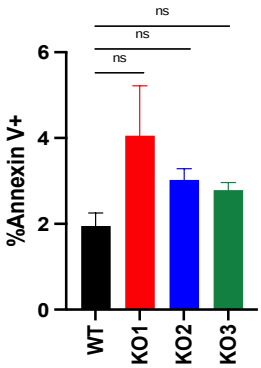
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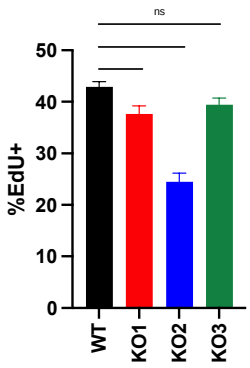
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HSF1 deficiency impairs AML progression and extends survival *in vivo*

Next, we investigated how the effects of HSF1 deficiency translate into AML progression *in vivo*. We adopted a bioluminescence imaging system to assess the leukemic burden *in vivo*. To do so, we performed lentiviral transduction to introduce luciferase into parental and HSF1 KO2 TF1a cells. To validate the system, we treated the cells with luciferin to produce luminescence. Transduced parental and HSF1 KO2 TF1a cells exhibited strong luminescence *in vitro* upon luciferin treatment, but the parental TF1a cells had 4.3-fold higher baseline luciferase activity (Figure S1A).

To generate an AML xenograft model, we transplanted the luciferase-expressing parental and HSF1 KO2 TF1a cells into immunocompromised NOD-scid IL2Rγ^{null} (NSG) mice via intravenous tail injection (Figure 5A). AML progression in transplanted mice was evaluated weekly by luciferin injection followed by *in vivo* bioluminescence imaging. To account for the baseline difference in luciferase activity *in vitro*, the bioluminescence intensity in recipients of parental TF1a cells was normalized by dividing by 4.3. HSF1 deficiency significantly reduced the leukemic burden *in vivo* (Figure 5B-C and S1B). Moreover, HSF1 deficiency significantly (P=0.0016) prolonged the survival of the recipient mice from a median survival time of 44 days to 66 days (Figure 5D). Taken together, these findings indicate that HSF1 promotes the growth and progression of AML *in vivo*.

Figure 5. HSF1 impairs AML progression and extends survival in vivo

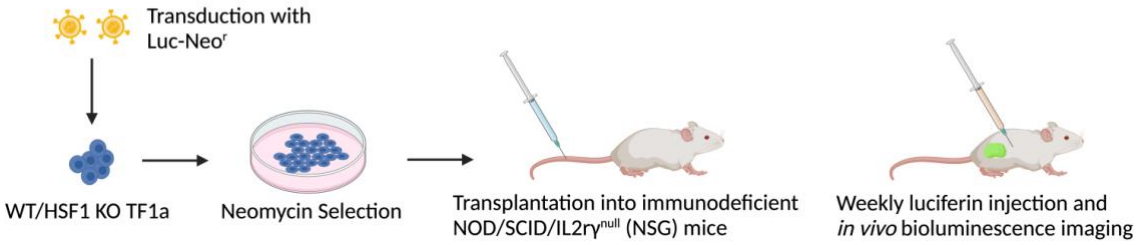
(A) Schematic figure of lentiviral transduction of parental (WT) and HSF1 KO TF1a cells to express luciferase, xenograft generation, and *in vivo* leukemia burden detection.

(B) Luciferase activity detected by *in vivo* bioluminescence imaging from day 21 to day 42 after transplantation of parental (WT) and HSF1 KO2 TF1a cells. (n=19 for WT, n=20 for KO)

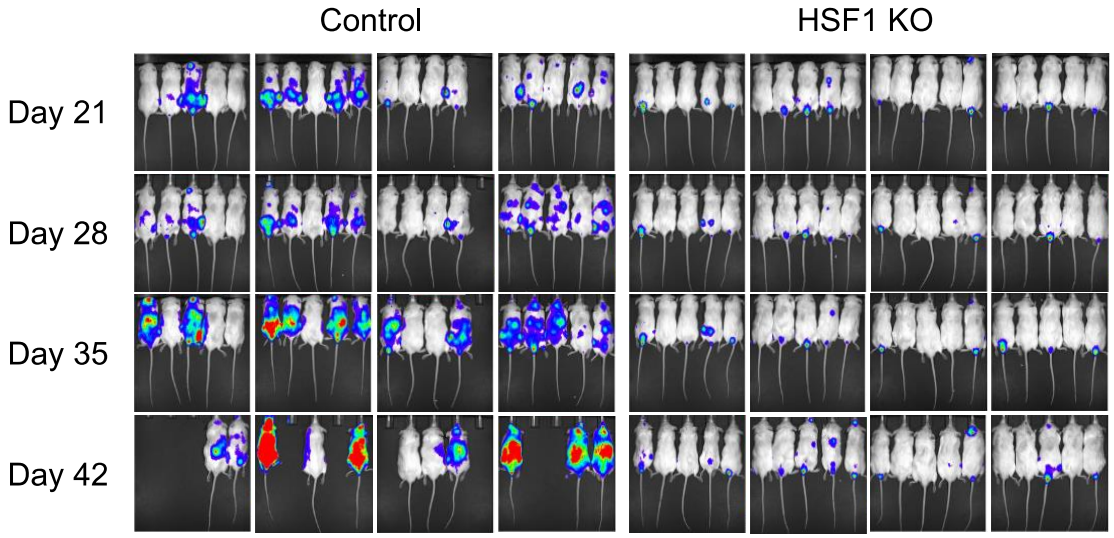
(C) Quantified luminescence of (B). The luminescence values of the control group were normalized by the luminescence difference (4.318:1) between the parental and HSF1 KO TF1a in vitro. Data represent mean ± S.E.M. Significance was assessed using a t test (*: $p<0.05$, **: $p<0.01$, ***: $p<0.001$).

(D) Survival curve of mice transplanted with parental (WT) and HSF1 KO TF1a cells.

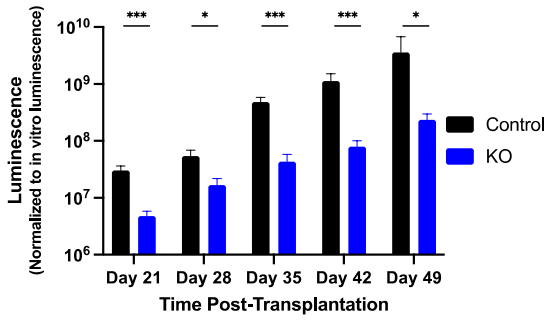
A


Transduction with
Luc-Neo^r
WT/HSF1 KO TF1a
Neomycin Selection
Transplantation into immunodeficient
NOD/SCID/IL2rγ^{null} (NSG) mice
Weekly luciferin injection and
in vivo bioluminescence imaging

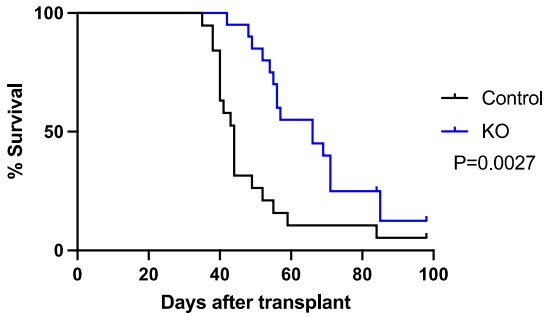
B


Control
HSF1 KO
Day 21
Day 28
Day 35
Day 42

C


Luminescence
(Normalized to in vitro luminescence)
10^{10}
10^9
10^8
10^7
10^6
***
*
***
***
*
Day 21
Day 28
Day 35
Day 42
Day 49
Time Post-Transplantation
Control
KO

D


% Survival
100
50
0
0
20
40
60
80
100
Days after transplant
Control
KO
P=0.0027

HSF1 deficiency does not increase proteotoxic stress in AML *in vitro*

Given the potent anti-leukemic effects of HSF1 depletion, we sought to gain additional mechanistic insights into the role of HSF1 in AML. As the master transcription factor regulating the heat shock response, HSF1 is a key regulator of proteostasis that can influence protein synthesis[77], folding, and clearance[69,78]. Hence, we hypothesized that HSF1 deficiency impaired AML growth by inducing proteotoxic stress. To test this, we performed a suite of proteostasis assays in parental and HSF1 KO TF1a cells to evaluate the impact of HSF1 depletion on protein synthesis as well as misfolded and unfolded protein abundance. Surprisingly, HSF1 loss was not associated with significant changes in these proteostasis parameters. HSF1 deficiency modestly reduced protein synthesis rates and increased the abundance of polyubiquitinated protein, a surrogate used to quantify misfolded proteins[24], but these changes did not reach the statistical significance threshold (Figure 6A-B). The abundance of unfolded proteins, quantified using tetraphenylethene maleimide (TMI)[79], was significantly increased in HSF1 KO2 cells, but not in other HSF1 KO TF1a cell clones (Figure 6C). These data suggest that HSF1 deficiency likely impairs AML cell growth *in vitro* through alternative mechanisms.

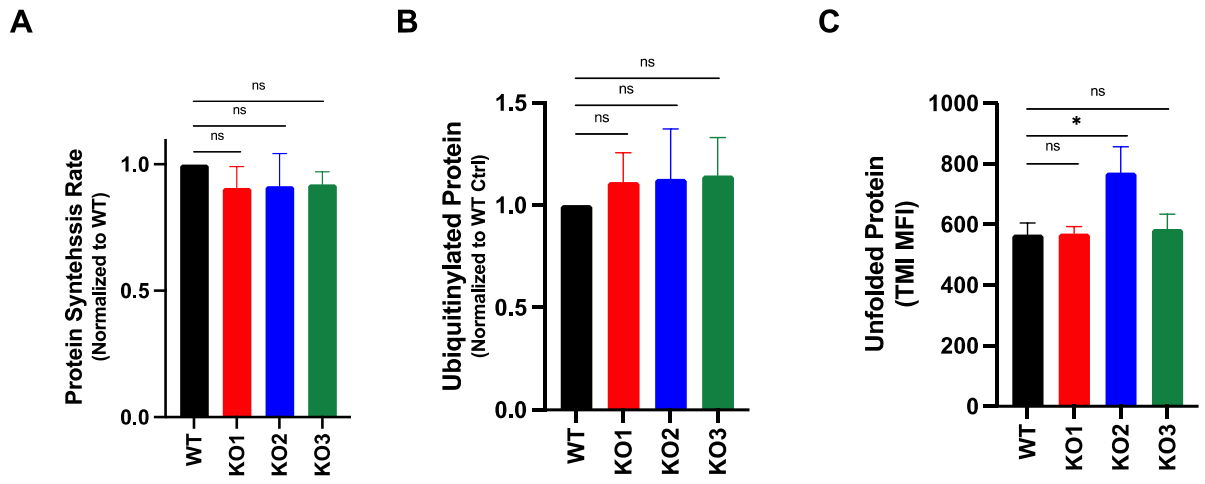


Figure 6. HSF1 deficiency does not increase proteotoxic stress in AML *in vitro*

(A) Relative protein synthesis measured by the incorporation of OP-Puro into parental (WT) and HSF1 KO TF1a cells (n=5).

(B) Relative abundance of polyubiquitinated proteins in parental (WT) and HSF1 KO TF1a cells (n=4).

(C) Relative abundance of unfolded proteins in parental (WT) and HSF1 KO TF1a measured by TMI fluorescence (n=4). Data represent mean ± S.E.M. (A-C) Cells were cultured at 10^6 cells/ml in duplicates (n=3). Significance was assessed using a t test (n.s: not significant, *: $p<0.05$)

HSF1 deficiency dysregulates the expression of genes involved in oncogenic pathways and translation

While HSF1 plays a significant role in proteostasis, it can also promote cancer progression through a variety of other cellular functions, including metabolism and signaling[67]. To better understand the role of HSF1 in AML, we performed RNA-sequencing of parental and HSF1 KO2 to identify differentially expressed genes and pathways. Principal component analysis confirmed that the variance in the gene expression data was primarily due to the presence of HSF1 (Figure 7A). HSF1 KO2 cells exhibited significant upregulation of 104 genes and downregulation of 523 genes (Figure 7B). More specifically, a gene set indicative of the ERK/MAPK pathway was notably repressed while a gene set representing the tumor suppressive PTEN pathway was activated (Figure 7C-E). In addition, gene sets for the eIF2 signaling pathway and translation were both upregulated in HSF1 KO2 TF1a cells (Figure 7C-D, F). These data raise the possibility that the dysregulation of oncogenic pathways and translation may underlie the anti-leukemic effects of HSF1 depletion.

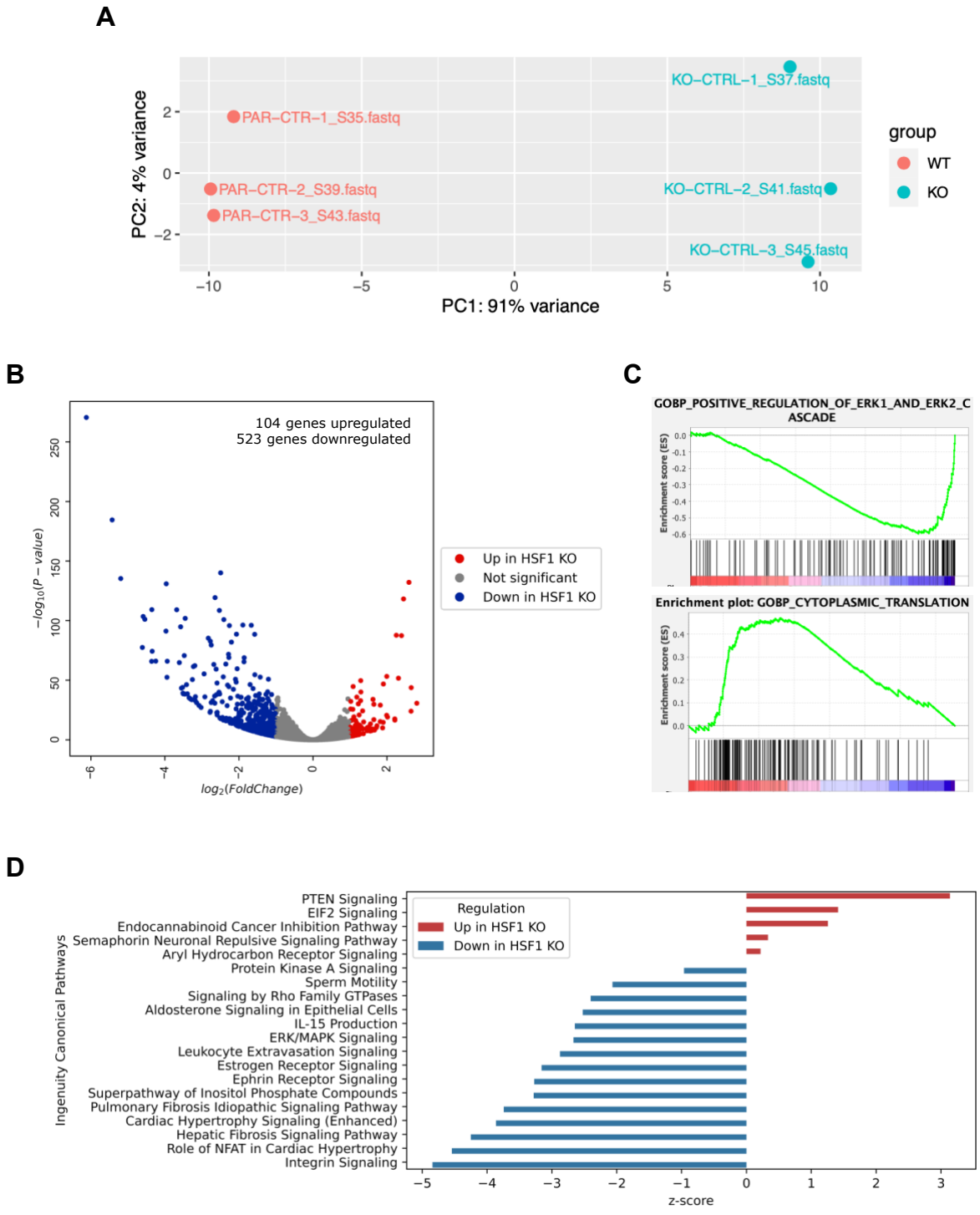


Figure 7. HSF1 deficiency dysregulates the expression of genes involved in oncogenic pathways and translation

(A) Principal component analysis of gene expression profiling in parental (WT) and HSF1 KO2 (KO) TF1a cells.

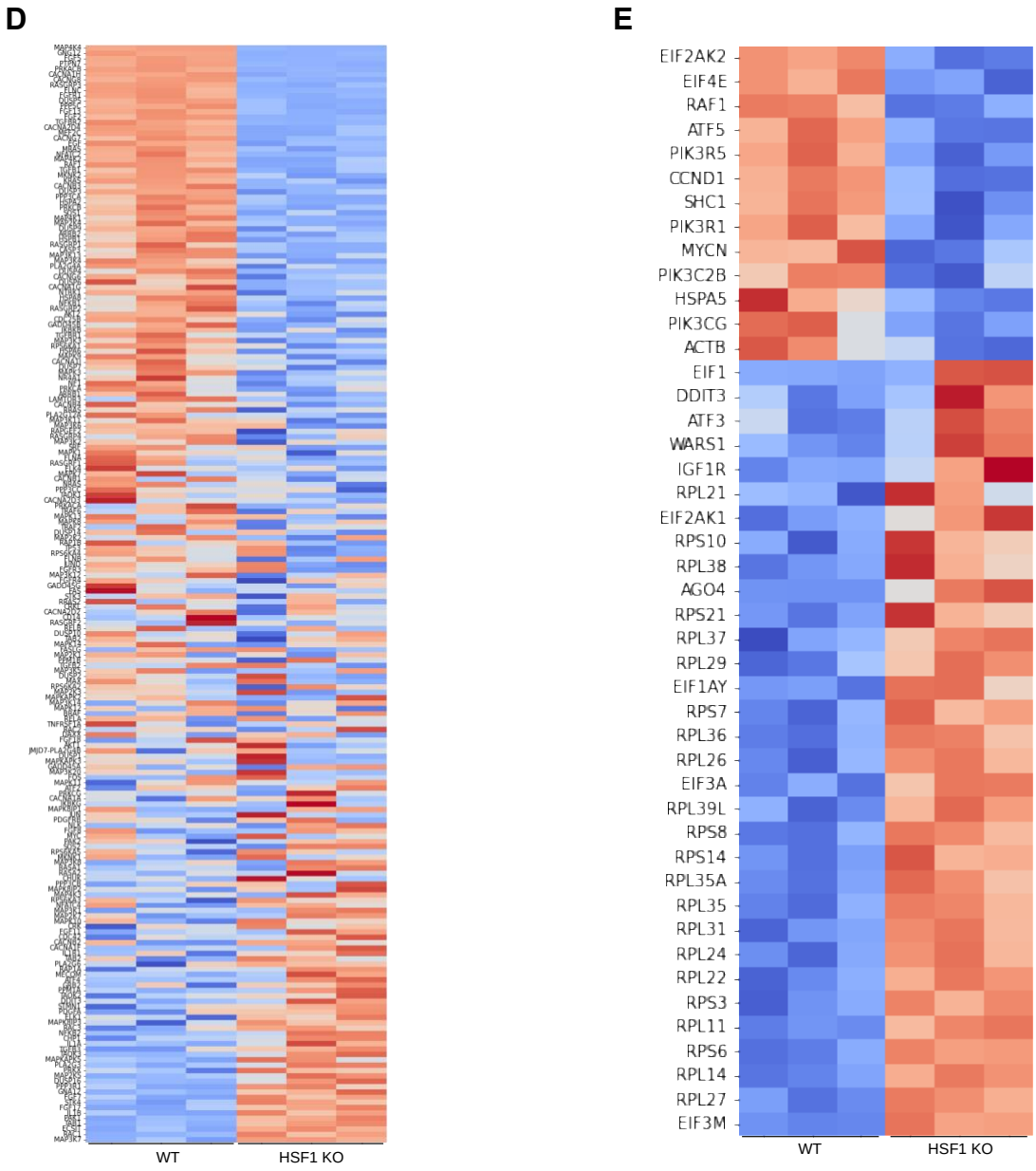


Figure 7. HSF1 deficiency dysregulates the expression of genes involved in oncogenic pathways and translation (continued)

(B) Volcano plot representation of differentially expressed genes between parental (WT) and HSF1 deficient (KO2) TF1a. Differentially expressed genes were defined as p-adj < 0.05 and abs($\log_2$(FC))>1.

(C) GSEA enrichment plot focusing on ERK regulation and translation pathways.

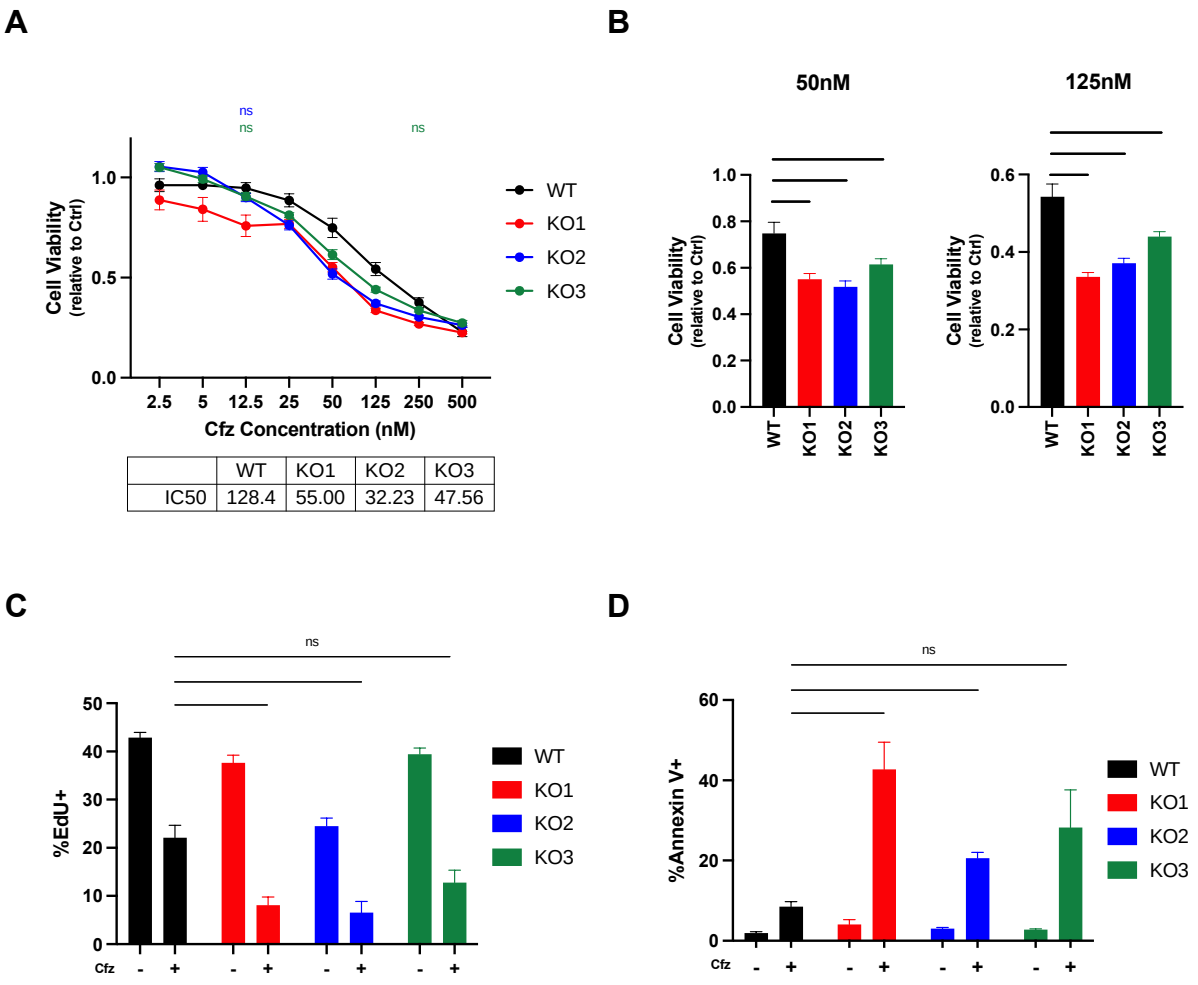
(D) Top 20 differentially regulated canonical pathways determined by Ingenuity Pathway Analysis.

(E) Heatmap representing the expression of genes involved in KEGG_MAPK_SIGNALING_PATHWAY.

(F) Heatmap representing the expression of genes involved in the eIF2 signaling pathway from Qiagen Ingenuity Pathway Analysis.

HSF1 deficiency increases AML sensitivity to proteasome inhibition *in vitro*

Although HSF1 deficiency on its own did not appear to induce significant proteotoxic stress, RNA-sequencing analysis indicated that some proteostasis pathways are perturbed. Furthermore, since HSF1 largely regulates proteostasis in response to stress, we hypothesized that HSF1 deficient AML cells may be increasingly sensitive to drugs that disrupt proteostasis. The most characterized cancer drugs that disrupt proteostasis are proteasome inhibitors, which are approved to treat multiple myeloma and mantle cell lymphoma[23,80]. To determine whether HSF1 deficiency sensitizes AML to further proteotoxic stress, we treated parental and HSF1 KO TF1a cells with varying doses of the proteasome inhibitor carfilzomib for 24h and assessed their viability. HSF1 KO TF1a cells treated with carfilzomib exhibited significantly reduced viability as compared to parental TF1a cells. HSF1 deficiency reduced carfilzomib IC50 values by 2.33- to 3.98-fold (Figure 8A-B). Furthermore, carfilzomib treatment had enhanced effects in reducing proliferation and increasing apoptosis in HSF1 KO TF1a cells compared to parental controls (Figure 8C-D). These data indicate that HSF1 deficiency sensitizes AML cells to carfilzomib treatment and suggests that simultaneous HSF1 and proteasome inhibition could be effective in targeting AML cells.



	WT	KO1	KO2	KO3
IC50	128.4	55.00	32.23	47.56

Figure 8. HSF1 deficiency increases AML sensitivity to proteasome inhibition *in vitro*

(A) Relative viability of parental (WT) and HSF1 KO TF1a cells treated with the indicated carfilzomib concentrations for 24 hours and the IC50 values for each cell line. Cells were cultured at $2.5x10^5$ cells/ml and treated in triplicates (n=4).

(B) Relative viability of parental (WT) and HSF1 KO TF1a cells treated with 50nM and 125nM carfilzomib for 24 hours. Cells were cultured at $2.5x10^5$ cells/ml and treated in triplicates (n=4).

(C) Frequency of EdU+ dividing parental (WT) and HSF1 KO TF1a cells treated with PBS control (-) and 125nM carfilzomib for 24 hours. Cells were cultured at 10^6 cells/ml and treated in duplicates (n=3).

(D) Frequency of annexin V+ apoptotic parental (WT) and HSF1 KO TF1a cells treated with PBS control (-) and 125nM carfilzomib for 24 hours. Cells were cultured at 10^6 cells/ml and treated in duplicates (n=3). (A-D) Data represent mean ± S.E.M. Significance was assessed using a t test (n.s: not significant, *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$).

Cysteamine treatment partially recapitulates the anti-leukemic effects of HSF1 deletion

Pharmacological targeting of HSF1 has drawn significant interest as a novel cancer therapy. However, direct inhibition of HSF1 has faced significant challenges, as transcription factors often do not harbor well-defined small-molecule binding sites[81]. Alternatively, inactivation of HSF1 by targeting its associated pathways has been investigated. Rohinitib can suppress HSF1 activation by inhibiting eIF4a and reducing translation initiation, and exhibits some anti-leukemic effects by decreasing AML growth and increasing sensitivity to FLT3 inhibitors[75]. Similarly, an enzyme that can be targeted to inactivate HSF1 is type 2 Transglutaminase (TG2). TG2 is a multifunctional enzyme that performs Ca^{2+} dependent post-translational modifications of proteins[82]. Through its protein disulfide isomerase (PDI) activity, TG2 mediates the trimerization of HSF1 required for its activation by catalyzing the intermolecular disulfide bond formation between Cys36 and Cys103[82]. Recently, it has been shown that cysteamine, a small molecule compound approved for cystinosis treatment, inhibits the PDI activity of TG2 and suppresses HSF1 activation[83,84]. Hence, we hypothesized that cysteamine treatment could recapitulate some of the anti-leukemic effects of HSF1 deletion.

To test this, we first evaluated the dose response of parental and HSF1 KO TF1a cells to a wide range of cysteamine concentrations. Cysteamine reduced cell viability in TF1a cells, but the effect was significantly diminished by HSF1 deficiency (Figure 9A-B). These data suggest that the anti-leukemic effect of cysteamine occurs at least partially by suppressing HSF1 activity. Finally, we assessed if cysteamine, similar to HSF1 deficiency, could sensitize AML cells to carfilzomib mediated proteasome inhibition. Indeed, the combined treatment of AML cells with cysteamine and carfilzomib produced a supra-additive effect in reducing AML viability, demonstrating the potential of the combination as a therapeutic agent for AML (Figure 9C).

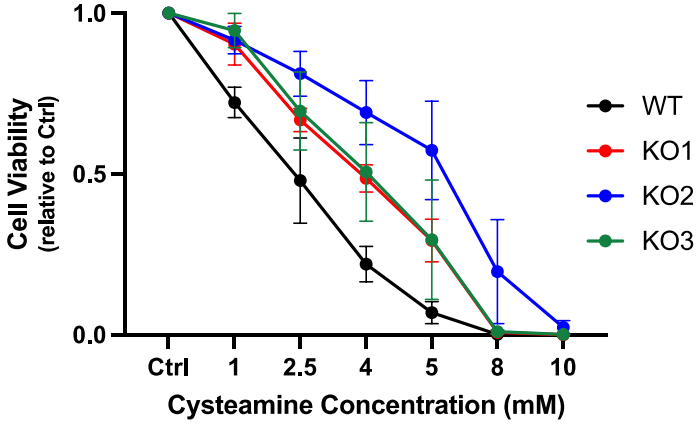
Figure 9. Cysteamine treatment partially recapitulates the anti-leukemic effects of HSF1 deletion

(A) Relative viability of parental (WT) and HSF1 KO TF1a cells treated with the indicated concentrations of cysteamine for 24 hours and the IC50 values of each cell line. Data represent mean ± S.E.M.

(B) Relative viability of parental (WT) and HSF1 KO TF1a cells treated with 4mM and 5mM cysteamine for 24 hours. Data represent mean ± S.E.M.

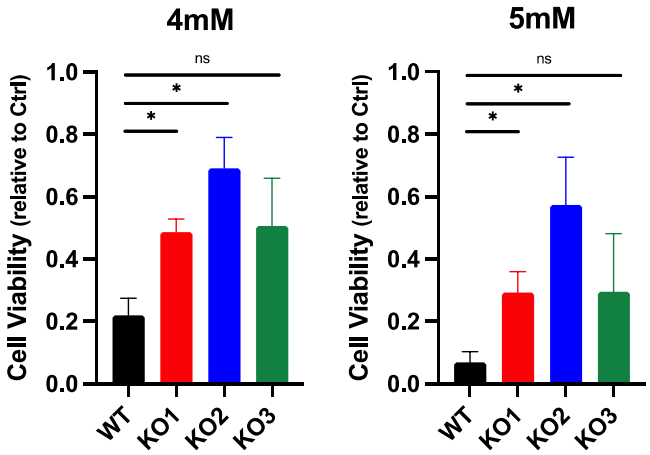
(C) Relative viability of parental TF1a cells treated with the indicated concentrations of cysteamine (Cys) and carfilzomib (Cfz) for 24 hours.

A

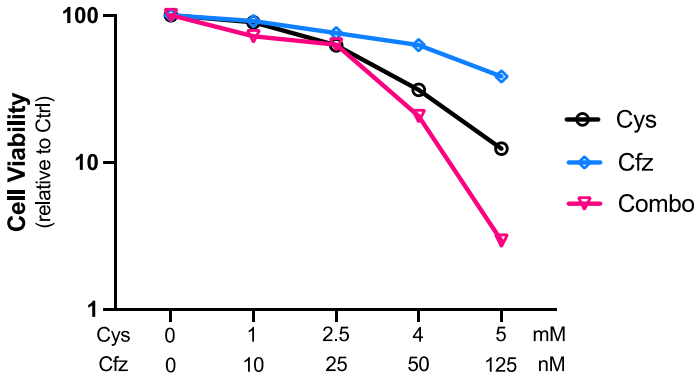


	WT	KO1	KO2	KO3
IC50	3.075	4.447	8.338	4.368

B



C



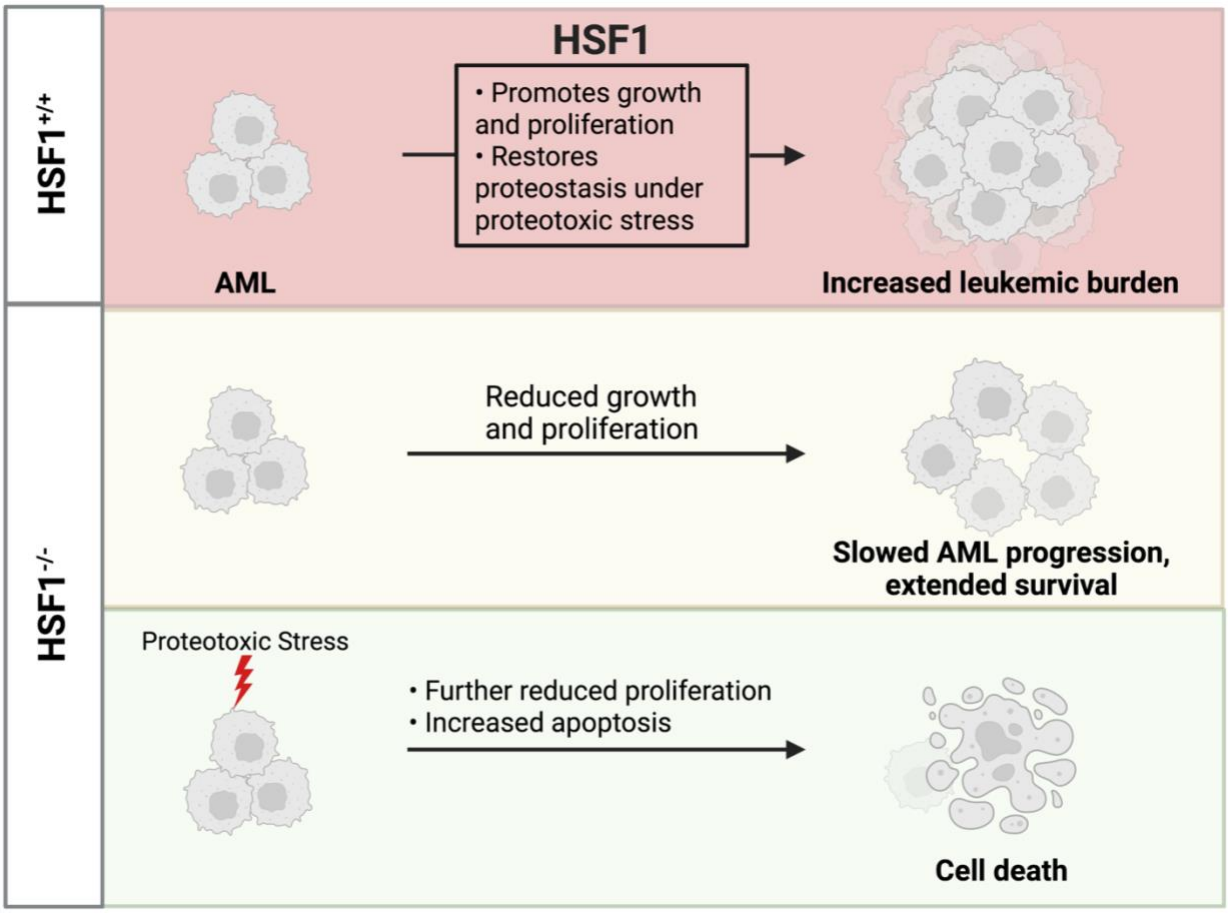


Figure 10. Schematic depiction of the role of HSF1 in AML growth and progression.
HSF1 deletion reduces AML growth and proliferation *in vitro*. HSF1 deficiency slows AML progression and extends survival *in vivo*. While HSF1 deletion does not directly produce proteotoxic stress in AML, HSF1 deficiency sensitizes AML cells to proteostasis disruption, enhancing the anti-proliferative and pro-apoptotic effects of proteasome inhibitor treatment. Together, these data suggest that HSF1 has a critical role in supporting AML growth and proliferation in addition to regulating proteostasis under proteotoxic stress.

DISCUSSION

Through its involvement in proteostasis, translation, metabolism, signaling, cell cycle, cell death, and immune response, HSF1 is a key stress response gene that can promote the survival and proliferation of malignant cells[67]. Across various types of cancers, HSF1 has been sought after as a therapeutic target and it has shown encouraging effectiveness in tumor suppression and therapy sensitization[69–72,74,75]. However, the role and therapeutic potential of HSF1 have not been established in AML. This provided a rationale for investigating the effect of HSF1 inhibition on AML growth and progression.

Here, we showed that HSF1 depletion mediated by CRISPR-Cas9 significantly reduced AML growth and proliferation *in vitro*, and significantly slowed AML progression and extended survival *in vivo*. Proteostasis and RNA-seq analyses suggest that proteostasis is not the primary culprit behind the suppressive effects of HSF1 deletion in AML. Rather, HSF1 ablation notably diminishes the activity of the oncogenic proliferative ERK/MAPK pathway and activates the tumor suppressive PTEN pathway, suggesting signaling and metabolic defects may underlie the anti-leukemic effects of HSF1 deletion. A key future direction is to continue to mechanistically unravel how HSF1 promotes AML growth.

Although HSF1 deficiency did not produce direct proteotoxic stress in AML cells, we found that HSF1 deficiency sensitized AML cells to proteostasis disruption. HSF1 deficient AML cells exhibited significantly increased sensitivity to the proteasome inhibitor carfilzomib, marked by reduced growth and proliferation as well as increased apoptosis. These data highlight the possibility that proteasome inhibitor treatments may be more effective in AML patients with low HSF1 expression. Combination therapy studies have still only been performed *in vitro*, but we plan

on conducting a carfilzomib treatment study on the xenograft AML models to evaluate the therapeutic potential of concurrent HSF1 and proteasome inhibition *in vivo*.

Recently, it was discovered that cysteamine, a TG2 inhibitor, can indirectly modulate HSF1 activity by mediating HSF1 trimerization[84]. Our results demonstrate that cysteamine produces anti-leukemic effects and that these effects at least partially depend on HSF1 as HSF1 deficient AML cells are significantly less sensitive to cysteamine treatment. Furthermore, cysteamine produces supra-additive effects when combined with carfilzomib, revealing a novel method to pharmacologically translate the therapeutic potential of concurrent HSF1 and proteasome inhibition. Nonetheless, there are several missing pieces to consolidate the anti-leukemic effects of the combination. To confirm that cysteamine is inhibiting HSF1 activity, we will be assessing the expression of downstream HSF1 transcriptional targets upon cysteamine treatment. Furthermore, we will perform an *in vivo* experiment to test the therapeutic efficacy and possible toxicity of cysteamine and carfilzomib combination treatment.

Although the results are in line with the published works on HSF1 inhibition, one limitation of our study is the variance of the phenotypes exhibited by the HSF1 KO clones. This inconsistency raises the question on if the effects we see are from HSF1 KO or off-target effects. To confirm that these observations are truly from HSF1 KO, HSF1 overexpression studies will be performed to assess if it rescues the growth and proliferation defects. Another limitation of our study is that the effect of HSF1 KO has only been evaluated in one AML cell line. Whether the effects of HSF1 depletion alone and in combination with proteasome inhibition can overcome the heterogeneity of AML remains to be elucidated with additional experiments on other AML cell lines and patient samples. Moreover, the xenograft model based on immunodeficient NSG mice neglects the immune response that is crucial to cancer therapy. As HSF1 can negatively regulate immune

responses[67], it is possible that HSF1 inhibition in AML may activate an immune response *in vivo* and produce a more distinct therapeutic efficacy. Alternatively, investigating the effects of HSF1 inhibition in genetically engineered immunocompetent mice that express leukemogenic proteins may better reflect the efficacy *in vivo* as well as its applicability to targeting LSCs.

Overall, these data identify a critical role for HSF1 in AML. Furthermore, these studies emphasize that the proteostasis network is highly integrated, and that compensatory mechanisms confer resistance to single agents targeting proteostasis activity. Inhibiting HSF1 thus holds enormous potential as a companion therapeutic for diverse approaches that target proteostasis in cancer.

MATERIALS AND METHODS

Cell culture and reagents

WT and HSF1 KO TF1a cells were cultured in RPMI-160 (Gibco) supplemented with 1% L-glutamine and 10% fetal bovine serum. MV4-11, OCI-AML2, and OCI-AML3 cells were cultured in IMDM (Gibco) supplemented with 1% L-glutamine and 10% fetal bovine serum. HEK293T cells were cultured in 10% fetal bovine serum supplemented DMEM (Gibco).

CRISPR-Cas9 mediated gene editing

To target HSF1, gRNAs were designed using the Benchling gRNA design tool. gRNAs from the first three exons were selected based on the highest off-target scores and higher on-target scores (Table 1). gRNA oligos were synthesized by IDT, *in vitro* annealed, and cloned into BbsI digested pSPCas9(BB)-2A-GFP (Addgene #48138) as previously described[85]. The plasmid was kindly provided by Dr. Xuezhen Ge (University of California San Diego, Department of Cell and Developmental Biology). The cloned plasmids were introduced into TF1a cells using Lipofectamine 3000 (Thermo Scientific) in 6 well plates according to the manufacturer's protocol. Transfected cells were sorted into 96-well plates at single cell resolution using FACSAria II (BD Biosciences). Following stable growth, target protein expression was assessed by Western blot.

Table 1. Sequence and location of the gRNAs used in CRISPR-Cas9 mediated HSF1 KO.

	Sequence	Location
gRNA1	GGTGTCCGGGTCGCTCACGA	Exon 1
gRNA2	ACTGGCCCTGGTCGAACACG	Exon 2
gRNA3	GTGGTCCACATCGAGCAGGG	Exon 3

Western Blot

Cells were prepared using lysis buffer consisting of RIPA buffer (Thermo Scientific) and protease inhibitor. Protein lysates were quantified using BCA protein assay kit (Pierce; Thermo Scientific). LDS loading buffer (Thermo Scientific) was added to 10-20µg proteins, heated at 98°C for 5 minutes, and centrifuged at 12,000g at 4°C for 1 minute. Samples were separated on 4-12% Bis-Tris gels (Life Technologies) and transferred to PVDF membranes (Bio-Rad). HSF1 (D3L8I; Cell Signaling 12972), phospho-HSF1 (S326; Abcam 76076), GAPDH (14C10; Cell Signaling 2118), and HRP-linked anti-rabbit IgG (Cell Signaling 7074) antibodies were used for probing. Blots were developed with the SuperSignal West Femto or Pico PLUS chemiluminescent substrate kits (Thermo Scientific) and stripped with 1% SDS, 25mM glycine (pH2) as needed.

Lentiviral Transduction

Lentivirus production and cell line transduction were done as previously described[86]. Briefly, HEK293T cells were mixed and transfected with 10µg Lenti-luciferase-P2A-Neo (Addgene #105621), 7.5µg psPAX2 (Addgene #12260), 5µg pMD2.G (Addgene #12259), and 40µl 1mg/ml polyethylenimine (Polysciences 23966-1). Viral supernatant was added to WT and HSF1 KO2 TF1a cells with 4µg/ml polybrene. Cells were centrifuged at 2000rpm at 32°C for 90 minutes.

In vitro experiments

Carfilzomib and cysteamine treatment

Cells were seeded in 150µl per well in 96-well plates at the density of $1x10^6$ cells/ml. Cells were treated with 150µl of 4% PBS in RPMI-1640 for control or indicated concentration of carfilzomib (2.5, 5, 12.5, 25, 50, 125, 250, and 500nM) and/or cysteamine (1/2.5/4/5/8/10mM).

Carfilzomib was reconstituted in PBS and 10mM stock was serially diluted in RPMI-1640 for treatment. Cysteamine (Sigma Aldrich) was reconstituted in DMSO and 1M stock was serially diluted in RPMI-1650 for treatment.

Cell growth measurement

WT and HSF1 KO TF1a cells were seeded in 1.5ml per well in 6-well plates at the density of $0.2x10^6$ cells/ml. Cells were counted every 24 hours upon plating using a hemocytometer via Trypan blue exclusion method to evaluate growth.

Cell viability assay

3-(4, 5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay kit (Roche) was used to evaluate cell viability. Briefly, cells were plated in 50µl per well in 96-well plates at the density of $5x10^5$ cells/ml. 50µl control or carfilzomib treatment was added to the wells to a final volume of 100µl/well. After 24-hour incubation at 37°C, 10µl MTT labeling reagent was added to each well and 100µl solubilization buffer was added to the wells 2 hours after labeling. The plates were incubated in 37°C overnight and 570nM absorbance was measured against 650nM reference absorbance using a microplate reader (Tecan).

Annexin V/PI apoptosis assay

FITC Annexin V Apoptosis Detection Kit (BD Biosciences) was used to detect apoptosis following a modified protocol based on the manufacturer's procedures. Briefly, cells were plated and treated as described above. 100µl cells were transferred to 5ml round bottom tubes and washed twice with PBS. Antibody cocktail containing 1µl FITC Annexin V and/or 1µl propidium iodide was added to the cells and incubated at room temperature in the dark for 15 minutes. Stained cells were then analyzed within an hour by flow cytometry.

Measurement of proliferation and protein synthesis

5-ethynyl-2'-deoxyuridine (EdU) and O-propargyl puromycin (OP-Puro) were used to assess cell proliferation and protein synthesis, respectively. Cells were plated and treated as described in the treatment protocol. 1µl of 10uM EdU or OP-Puro was added to the wells 24 hours after treatment and incubated at 37°C for 1 hour. 100µl cells were transferred to 5ml round bottom polystyrene tubes and fixed with 500µl 1% paraformaldehyde for 10 minutes on ice. Cells were then washed with PBS and permeabilized with PBS supplemented with 0.1% saponin (Sigma Aldrich) and 3% heat inactivated fetal bovine serum (Gibco) for 5 minutes at room temperature. 65µl Click-iT cocktail was added to the cells and incubated at room temperature away from light for 30 minutes to perform the azide-alkyne cycloaddition using the Click-iT Reaction Buffer Kit (Invitrogen C10269) and azide-conjugated Alexa Fluor 555 (Life Technologies A20012). Cells were washed with the permeabilization buffer described above, resuspended in PBS supplemented with DAPI (4mg/ml final concentration), and analyzed by flow cytometry.

Measurement of ubiquitinated proteins

Cells were plated, treated, transferred, fixed with 1% paraformaldehyde, washed with PBS, and permeabilized as described above. Cells were stained with 1:500 anti-ubiquitinylated protein FK2 antibody (Sigma Aldrich 04-263) in HBSS supplemented with 2% heat inactivated fetal bovine serum for 30 minutes at room temperature protected from light and washed with the permeabilization buffer described above. This was followed by incubation with 1:500 anti-mouse Alexa Fluor 488 (Life Technologies A-11029) for 30 minutes at room temperature in the dark. Cells were then washed with the permeabilization buffer, resuspended in PBS supplemented with DAPI (4mg/ml final concentration), and analyzed by flow cytometry.

Measurement of unfolded proteins

Cells were plated and treated as described above. Cells were transferred to round bottom 5ml tubes and washed with PBS. 60µl of 50uM tetraphenylethene maleimide (TMI; stock 2 mM in DMSO) dissolved in PBS was added to the samples and incubated at 37°C for 45 minutes. Samples were washed with PBS, resuspended in PBS, and analyzed by flow cytometry.

RNA-Seq

For RNA-seq analysis, total RNA was extracted from 10^6 cells using the RNeasy Plus Micro Kit (Qiagen). Illumina mRNA libraries were prepared using the SMARTseq2 protocol and sequenced in two lanes on the Illumina HiSeq 2500 by the Sequencing core at the La Jolla Institute of Immunology. The reads were trimmed using TrimGalore and aligned against the human genome assembly GRCh38 using HiSAT2. Gene counts were subsequently obtained using FeatureCounts and differentially expressed genes were obtained using DESeq2. P-values for differential expression are calculated using the Wald test for differences between the base means of two conditions. These P-values are then adjusted for multiple test correction using Benjamini Hochberg algorithm. GSEA was run for the HALLMARK, GO_BP, and KEGG gene sets, and pathways were analyzed using Qiagen Ingenuity Pathway Analysis software.

***In vivo* experiments**

An equal number of male and female NSG mice (6-8 weeks old; Jackson Laboratory) were intravenously injected with luciferase-bearing WT or HSF1 KO TF1a cells (0.5 x 10^6 cells per mouse; n=19 for WT, n=20 for HSF1 KO). Xenograft mice were injected with D-luciferin (150mg/kg bodyweight; PerkinElmer) and luciferase activity was measured using the IVIS200 *in*

vivo imaging system (PerkinElmer) from week 3 post-transplantation until the mice were moribund.

APPENDIX

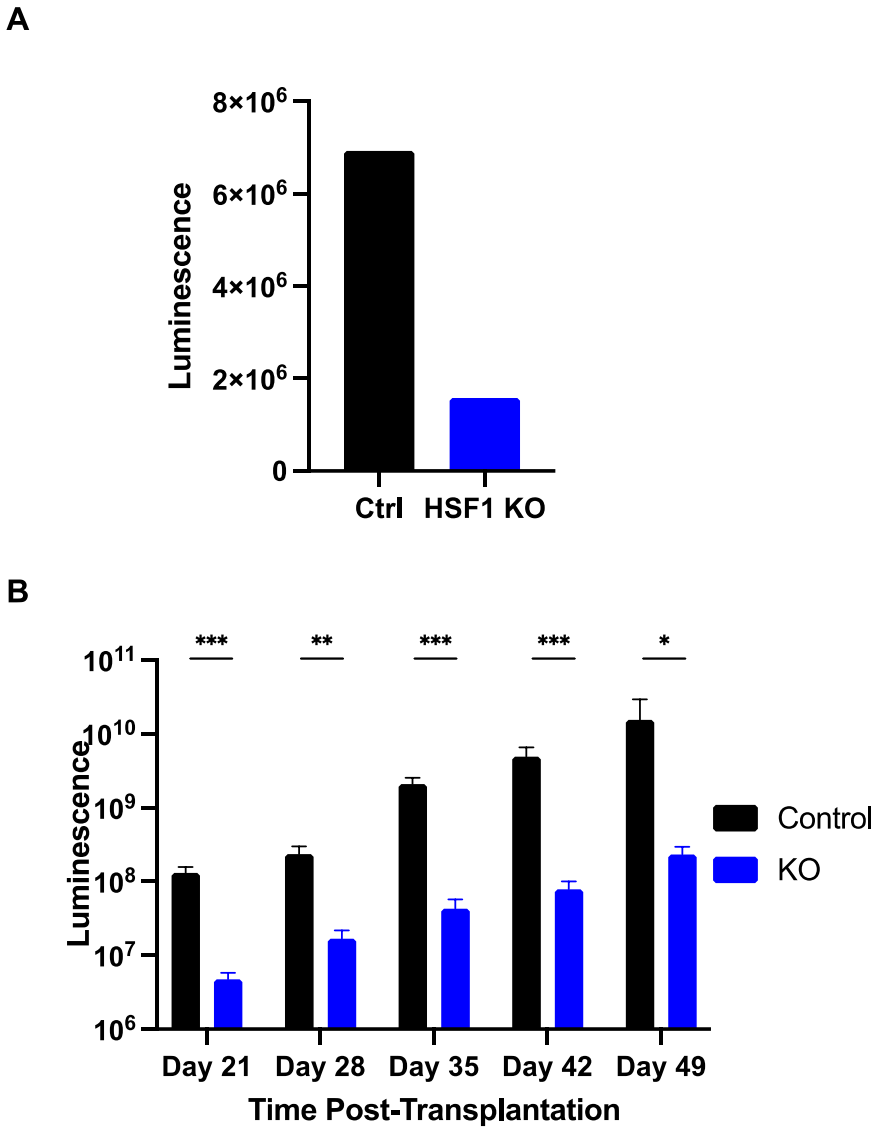


Figure S1. Luciferase transduced parental TF1a cells have higher luminescence than HSF1 KO cells in vitro.

(A) Quantified bioluminescence of luciferase transduced parental (Ctrl) and HSF1 KO2 (KO) TF1a cells. 100ul cells were plated at 10^6cells/ml and lysed with Luciferase Cell Culture Lysis Reagent to measure the bioluminescence (n=1).

(B) Raw quantified luminescence of mice transplanted with luciferase transduced parental (Control) and HSF1 KO2 (KO) TF1a cells. Data represent mean ± S.E.M. Significance was assessed using a t test (*: $p<0.05$, **: $p<0.01$, ***: $p<0.001$). n=19 for control, n=20 for KO.

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