Combined chemical–genetic approach identifies cytosolic HSP70 dependence in rhabdomyosarcoma

Amit J. Sabnis[a,b,1], Christopher J. Guerriero[c,1], Victor Olivas[b,d], Anin Sayana[d], Jonathan Shue[d], Jennifer Flanagan[d], Saurabh Asthana[b,d], Adrienne W. Paton[e], James C. Paton[e], Jason E. Gestwicki[f], Peter Walter[g,h], Jonathan S. Weissman[h,i], Peter Wipf[j], Jeffrey L. Brodsky[c], and Trever G. Bivona[b,d,2]

[a]Department of Pediatrics, University of California, San Francisco, CA 94143; [b]Helen Diller Family Comprehensive Cancer Center, University of California, San Francisco, CA 94143; [c]Department of Biological Sciences, University of Pittsburgh, Pittsburgh, PA 15260; [d]Department of Medicine, University of California, San Francisco, CA 94143; [e]Research Centre for Infectious Diseases, School of Biological Sciences, University of Adelaide, Adelaide 5005, Australia; [f]Institute for Neurodegenerative Disease, University of California, San Francisco, CA 94143; [g]Department of Biochemistry and Biophysics, University of California, San Francisco, CA 94143; [h]Howard Hughes Medical Institute, University of California, San Francisco, CA 94143; [i]Department of Cellular and Molecular Pharmacology, University of California, San Francisco, CA 94143; and [j]Department of Chemistry, University of Pittsburgh, Pittsburgh, PA 15260

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Cytosolic and organelle-based heat-shock protein (HSP) chaperones ensure proper folding and function of nascent and injured polypeptides to support cell growth. Under conditions of cellular stress, including oncogenic transformation, proteostasis components maintain homeostasis and prevent apoptosis. Although this cancer-relevant function has provided a rationale for therapeutically targeting proteostasis regulators (e.g., HSP90), cancer-subtype dependencies upon particular proteostasis components are relatively undefined. Here, we show that human rhabdomyosarcoma (RMS) cells, but not several other cancer cell types, depend upon heat-shock protein 70 kDA (HSP70) for survival. HSP70-targeted therapy (but not chemotherapeutic agents) promoted apoptosis in RMS cells by triggering an unfolded protein response (UPR) that induced PRKR-like endoplasmic reticulum kinase (PERK)–eukaryotic translation initiation factor α (eIF2α)–CEBP homologous protein (CHOP) signaling and CHOP-mediated cell death. Intriguingly, inhibition of only cytosolic HSP70 induced the UPR, suggesting that the essential activity of HSP70 in RMS cells lies at the endoplasmic reticulum–cytosol interface. We also found that increased CHOP mRNA in clinical specimens was a biomarker for poor outcomes in chemotherapy-treated RMS patients. The data suggest that, like human epidermal growth factor receptor 2 (*HER2*) amplification in breast cancer, increased CHOP in RMS is a biomarker of decreased response to chemotherapy but enhanced response to targeted therapy. Our findings identify the cytosolic HSP70–UPR axis as an unexpected regulator of RMS pathogenesis, revealing HSP70-targeted therapy as a promising strategy to engage CHOP-mediated apoptosis and improve RMS treatment. Our study highlights the utility of dissecting cancer subtype-specific dependencies on proteostasis networks to uncover unanticipated cancer vulnerabilities.

HSP70 | chaperone | cancer | sarcoma | unfolded protein response

The synthesis and folding of proteins is a highly regulated process in both normal and malignant cells (1). The protein homeostasis ("proteostasis") network that operates in cancer cells offers targets for therapeutic development, such as the proteasome and specific protein chaperones (2, 3). For example, the heat-shock protein 90 kDa (HSP90) chaperone maintains the stability of some mutant oncoproteins and permits the outgrowth of drug-resistant cells, fueling the development of small molecule HSP90 inhibitors as anticancer agents (4). Despite promising early results, these HSP90 inhibitors do not show large-scale clinical success across the majority of cancers tested (4). Proteasome inhibitor treatment has made a substantial impact on cancer patient outcomes but to date has been highly effective in only a small number of malignancies (5). Although the proteostasis network provides an increasingly rich landscape beyond these two targets, the dependence of particular cancer subtypes on specific proteostasis components is not well defined. Filling this knowledge gap is essential to elaborate the role of proteostasis in the pathogenesis of different malignancies and to identify cancer-specific vulnerabilities for therapeutic exploitation.

Heat-shock protein 70 kDa (HSP70) is a chaperone that can facilitate tumor cell growth and is up-regulated in response to other protein homeostasis-targeted therapies, such as inhibitors of HSP90 and the proteasome (6, 7). Indeed, HSP70 induction likely lessens the therapeutic effects of such inhibitors (8). HSP70s maintain cellular homeostasis by binding misfolded polypeptides and, through a cycle of cochaperone-accelerated ATP hydrolysis, refold these clients, transfer them to HSP90, facilitate protein trafficking or posttranslational modifications, or target misfolded substrates for degradation (9). The human genome encodes 14 distinct HSP70 family members that have unique subcellular localizations, inducible or constitutive expression patterns, and/or activities. This specialization in function suggests that pharmacologic inhibition of HSP70 will provide a therapeutic window in certain cancer subsets. Recently discovered small molecule HSP70 inhibitors have shown some activity in select preclinical cancer models (10–14). The availability of these HSP70 inhibitors creates,

Significance

Protein chaperone networks maintain homeostasis during cellular stress. Oncogenic transformation induces stress through increased demands on protein synthesis and folding. Thus, many cancer cells depend on proteostasis networks for optimal growth. However, the cancer subtype-specific roles of individual protein chaperones are incompletely understood. Through a chemical–genetic approach, we discovered an exquisite dependence of rhabdomyosarcoma (RMS) cells on cytosolic heat-shock protein 70 kDa (HSP70). HSP70 inhibition activates the unfolded protein response, and CEBP homologous protein is a key mediator of apoptosis and a candidate biomarker for efficacy. The link between a component required for cytosolic protein quality control and the endoplasmic reticulum stress response provides insight into cell type-specific wiring of proteostasis networks and suggests novel therapeutic avenues in RMS.

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[1]A.J.S. and C.J.G. contributed equally to this work.

[2]To whom correspondence should be addressed. Email: trever.bivona@ucsf.edu.

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for the first time, an opportunity to understand the role of this critical proteostasis factor in cancer subtypes.

Sarcomas are connective tissue tumors that comprise a substantial burden of pediatric cancer incidence and mortality (15). A subset of pediatric sarcomas is driven by fusion oncogenes. Rhabdomyosarcoma (RMS), the most common soft tissue sarcoma affecting children and young adults, provides a compelling case for targeting fusion oncoproteins in these connective tissue malignancies. Although overall survival for children with RMS approaches 70%, the presence of the paired box 3-forkhead box O1 (*PAX3-FOXO1*) fusion strongly associates with adverse outcome predictors such as an extremity primary site and metastatic disease at diagnosis (16). Despite 30 y of clinical trials, *PAX3-FOXO1* fusion-positive RMS patients with metastatic disease are considered incurable with current therapies (17). Difficulties in directly inhibiting the *PAX3-FOXO1* transcription factor chimera and a largely unaltered genomic landscape in RMS have, to date, precluded precision medicine in this disease (18, 19). To test whether HSP70 represents an alternative therapeutic target in RMS, we explored the hypothesis that HSP70 is essential for RMS cell survival, either through stabilizing the PAX3-FOXO1 fusion protein or another function.

Results

HSP70 Function Is Essential for RMS Cell Viability. We used the tool compound MAL3-101 (20) to assess whether HSP70 chaperone function is required for survival in a panel of patient-derived, fusion-driven solid cancer models (Table S1). MAL3-101 is a specific HSP70 inhibitor that binds to an allosteric site within the chaperone's ATPase domain, thus inhibiting catalytic activation by J domain-containing HSP40 chaperones (21). We found that different fusion-driven cancer models were not uniformly sensitive to inhibition of HSP70 activity but rather that RMS cell lines exhibited a unique MAL3-101 sensitivity (Fig. 1*A*). Furthermore, the *PAX3-FOXO1*–negative RMS cell lines RD and Rh18 were also quite sensitive to MAL3-101 (Fig. 1*A*). Because MAL3-101 is a mixture of four stereoisomers, we also tested the toxicity of the isomers individually but found them to be comparable to the mixture (Fig. S1). The data reveal a unique dependence upon HSP70 activity for RMS cell growth and show that the growth-suppressive effects of inhibiting HSP70 activity are independent of the presence of PAX3-FOXO1.

In support of this notion, immunoblots of *PAX3-FOXO1*–positive RMS13 cells treated with MAL3-101 showed rapid induction of apoptosis, without substantial concurrent degradation of the PAX3-FOXO1 protein (Fig. 1 *B* and *D*). We independently confirmed that the growth suppression and apoptosis induced by MAL3-101 extended to *PAX3-FOXO1* fusion–negative RD cells (Fig. 1 *C* and *D*). Prior work has shown that HSP70 may stabilize C-RAF and AKT (22), thus sustaining survival signaling through the MAP kinase and PI3-kinase pathways. However, we found no evidence of substantial degradation of these proteins in the time course during which MAL3-101 induces apoptosis in RMS13 or RD cells (Fig. 1 *B*–*D*). Based on these results, we hypothesized that inhibiting HSP70 activation with MAL3-101 led to RMS cell death, not via the loss of well-documented cancer-survival signals such as PAX3-FOXO1, MAPK, or PI3K, but instead through collapse of a critical proteostasis network that is essential for RMS cell survival.

HSP70 Inhibition Activates the Unfolded Protein Response in RMS Cells. We next used an unbiased approach to identify the basis of the lethal effects of HSP70 inhibition by MAL3-101 in RMS cells. Perturbation of chaperone components can cause profound and specific changes in gene expression that dictate the cellular response to chaperone modulation (23). As such, we reasoned that transcriptome analysis might reveal a specific cellular program that is engaged by MAL3-101 treatment to cause RMS cell death. We found that DNA damage inducible transcript 3 (*DDIT3*), the gene encoding CEBP homologous protein (CHOP), was among the most strongly up-regulated genes following MAL3-101 treatment (Fig. 2*A*). Because CHOP has been associated with apoptosis downstream of the accumulation of unfolded proteins in the endoplasmic reticulum (ER) (24), our finding raised the possibility that HSP70 inhibition engages the unfolded protein response (UPR) and induces CHOP to kill RMS cells.

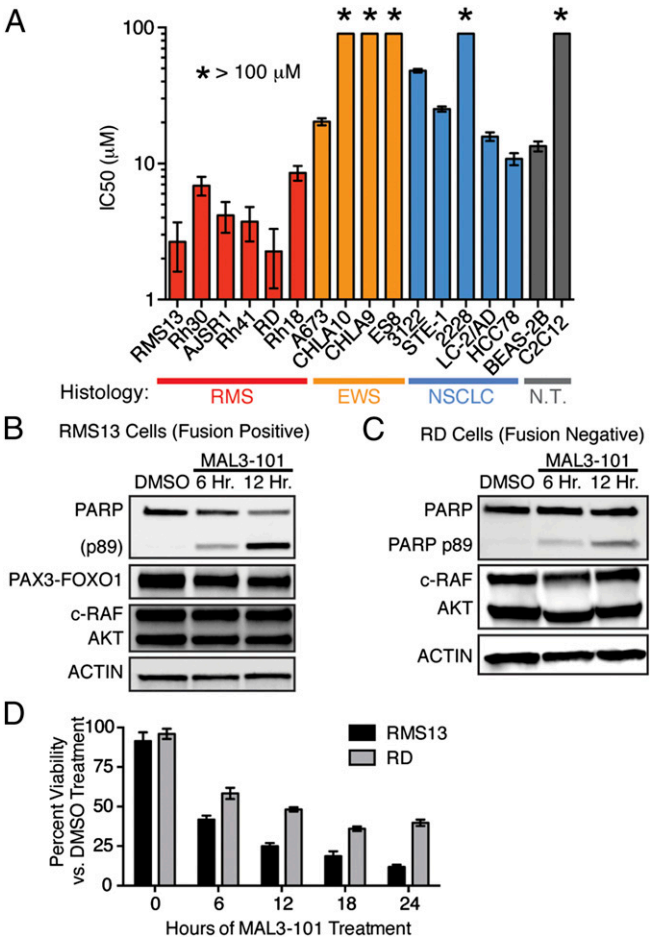

Fig. 1. Human RMS cell survival depends upon HSP70 activation. (*A*) Cell lines were seeded in 96-well plates and treated with increasing MAL3-101, an inhibitor of HSP70 cochaperone activation. Viability at 72 h was measured using a CellTiter-Glo assay; IC_{50} doses were calculated using nonlinear regression. Data are shown as mean ± SEM ($n = 3$). (*B* and *C*) RMS13 (*B*) and RD (*C*) cells were treated with 10 μM MAL3-101 for the indicated times, and whole-cell lysates were blotted for PARP cleavage, PAX3-FOXO1, c-RAF, AKT, and β-actin. Data represent three independent experiments. (*D*) RMS13 and RD cells were treated with 10 μM MAL3-101 or DMSO, and numbers of viable cells were counted by trypan blue exclusion at the indicated times. The mean percentage (± SEM) of viable cells compared with DMSO is shown ($n = 6$).

The UPR is a conserved network of signaling pathways that permit either an adaptive survival or apoptotic response to misfolded proteins in the ER (25). Three distinct UPR sensors, PRKR-like endoplasmic reticulum kinase (PERK), inositol-requiring enzyme 1 (IRE1), and activating transcription factor 6 (ATF6), have different transcriptional outputs in mammalian cells. PERK activation enhances transcription and translation of both activating transcription factor 4 (ATF4) and CHOP (25). Both Ingenuity Pathway Analysis (26) and gene set enrichment analysis (GSEA) (27, 28) of the gene expression changes induced by MAL3-101 treatment showed strong enrichment for genes that are direct targets of the CHOP and ATF4 transcription factors (Fig. 2 *A* and *B* and Table S2) (29). Further, we found that HSP70 inhibition with MAL3-101 biochemically engaged the PERK–eukaryotic

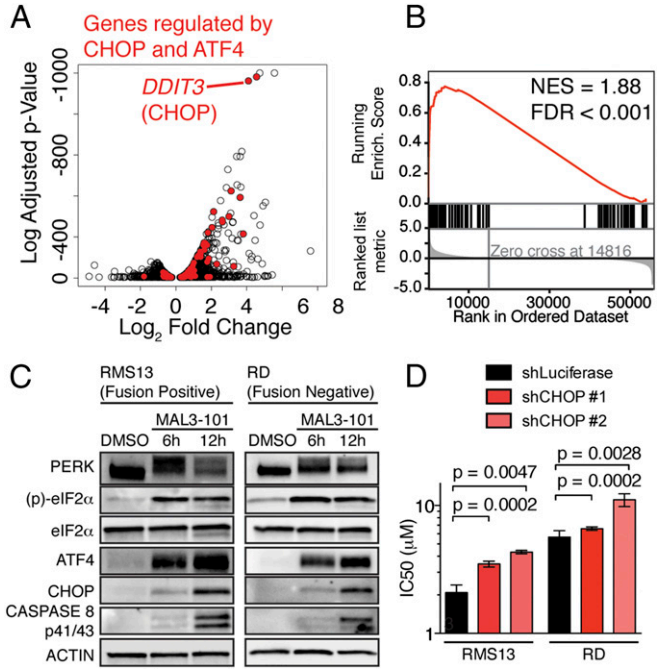

Fig. 2. HSP70 inhibition triggers the UPR and engages CHOP to promote RMS apoptosis. (*A*) Genes regulated by 9-h MAL3-101 versus DMSO treatment in RMS13 cells. The $\log_2$ fold-change is plotted against the $\log_{10}$-adjusted *P* value (Benjamini–Hochberg correction). Genes regulated by CHOP and ATF4 in mouse embryonic fibroblasts after tunicamycin (29) are highlighted in red, and CHOP (*DDIT3*) is indicated. (*B*) GSEA of the genes highlighted in *A*. NES, normalized enrichment score; FDR, false-discovery rate. (*C*) Immunoblots from RMS13 and RD cells treated with 10 μM MAL3-101, representing three independent experiments. Lysates were run on the same gel as in Fig. 1*B*, so the loading control is repeated. (*D*) RMS13 and RD cells were transduced with shRNAs against luciferase (control) or *DDIT3* (CHOP). The IC_{50} was measured as in Fig. 1*A*. Bar plots show mean ± SEM; *P* values were calculated by unpaired *t* test (n = 6–9).

translation initiation factor α (eIF2α)–CHOP signaling axis in RMS cells, as shown by electrophoretic mobility shift of PERK (consistent with phosphorylation and activation), phosphorylation of the PERK target eIF2α, and induction of ATF4 and CHOP in both PAX3-FOXO1–positive and PAX3-FOXO1–negative RMS cell lines (Fig. 2*C*). Additionally, we observed proteolytic cleavage and activation of caspase 8, consistent with recent reports indicating that caspase 8 promotes cell death downstream of CHOP (30).

Inhibition of HSP70 Leads to CHOP-Dependent Apoptosis. To test the importance of CHOP in inducing MAL3-101–dependent apoptosis, we used shRNAs to knock down *DDIT3* (CHOP) in fusion-positive RMS13 cells and fusion-negative RD cells. In both these systems, CHOP knockdown promoted MAL3-101 resistance (Fig. 2*D* and Fig. S2 *A*–*C*), suggesting that CHOP is required for cell death induced by MAL3-101 treatment. Consistent with these findings, CHOP overexpression partially reversed the effects of shRNA-mediated CHOP knockdown (Fig. S2*D*). This observation suggests that off-target shRNA effects are unlikely to explain the CHOP knockdown phenotype and that increased CHOP sensitizes RMS cells to HSP70 inhibition.

As an independent means to assess the role of CHOP after MAL3-101 treatment, we confirmed that overexpression of the C terminus of Chinese hamster GADD34 (31), a catalytic phosphatase subunit that dephosphorylates eIF2α and suppresses CHOP, similarly promoted MAL3-101 resistance (Fig. S2*E*). The degree of CHOP suppression achieved by these genetic manipulations correlated with the amount of poly(ADP-ribose) polymerase (PARP) cleavage observed upon MAL3-101 treatment; namely, decreased CHOP was associated with less PARP cleavage (Fig. S2*F*). Pharmacologic inhibition of the UPR sensor PERK (32) did not block CHOP induction after MAL3-101 treatment, despite blocking both PERK autophosphorylation and CHOP induction after the addition of tunicamycin (Fig. S2*G*). These data highlight the known functional redundancy among eIF2α kinases (33). Finally, cancer cell lines that were MAL3-101 resistant either had insufficient UPR activation to induce CHOP or failed to activate the UPR after MAL3-101 treatment (Fig. S2*H*). These collective data indicate that CHOP induction is necessary for MAL3-101–induced apoptosis.

Cytosolic HSP70s Are the Essential Targets of MAL3-101. We next asked whether toxic activation of the UPR by MAL3-101 was caused by inhibition of a specific HSP70 isoform. Because misfolded peptides in the ER lumen canonically activate ER-resident UPR sensors such as PERK, we reasoned that loss of the ER-localized HSP70, binding protein (BiP), would be sufficient to reproduce the drug's effects. To address this hypothesis, we treated RMS13 cells with the subtilase cytotoxin (SubAB), which proteolytically cleaves BiP and thereby abrogates its function (34), and asked whether this treatment recapitulates the biochemical and transcriptional effects of MAL3-101. To our surprise, SubAB did not induce apoptosis as measured by PARP cleavage within the same time course as MAL3-101 (Fig. 3*A*). Further, SubAB induced CHOP mRNA to only half the level of MAL3-101 despite complete clearance of BiP protein from the SubAB-treated cells (Fig. 3 *A* and *B*). By contrast, SubAB more strongly activated the UPR sensor IRE1 as indicated by dramatically higher splicing of *XBP1* in cells treated with SubAB than in cells treated with MAL3-101 (Fig. 3*B*). Finally, although inhibition of BiP with SubAB toxin induced a compensatory up-regulation of BiP mRNA (*HSPA5*), MAL3-101 instead provoked strong up-regulation of *HSPA1A*, which encodes the cytosolic chaperone HSP72 (Fig. 3*B*). Thus, inhibition of the ER-resident HSP70 isoform BiP leads to a transcriptional program and cell fate distinct from that seen with MAL3-101 treatment.

Based on the induction of HSP72 that we observed upon MAL3-101 treatment (Fig. 3*B*), we hypothesized that MAL3-101 predominantly targets a cytosolic chaperone (35). To test this hypothesis, we used a genetic approach. We found that knockdown of the inducible cytosolic chaperone HSP72 (encoded by *HSPA1A* and *HSPA1B*) had little effect on either UPR activation or cell viability (Fig. 3*C*). Knockdown of the major constitutively expressed cytosolic HSP70, HSC70 (*HSPA8*), produced strong eIF2α phosphorylation and ATF4 induction but minimal CHOP induction and only a minor loss of cell viability (Fig. 3*C*). This effect was accompanied by up-regulation of the stress-inducible HSP72 isoform (Fig. 3*C*). We reasoned that this compensatory induction of HSP72 provides sufficient chaperone activity to rescue cells from full commitment to UPR-mediated death. Therefore, we knocked down HSP72 (*HSPA1A* and *HSPA1B*) and HSC70 (*HSPA8*) concomitantly and found that combined knockdown of the inducible and constitutive forms of cytosolic HSP70 provoked robust UPR activation accompanied by PARP cleavage and loss of RMS cell viability (Fig. 3*C*). Additionally, simultaneous knockdown of HSP72 and HSC70 sensitized both fusion-positive and fusion-negative RMS cells to MAL3-101 (Fig. S3), as was consistent with depletion of the relevant drug target(s).

As an alternative approach to identify the relevant target of MAL3-101 in RMS cells, we grew RMS13 cells under a steady dose escalation of the compound and clonally derived resistant (RMS13-R) populations. The isolated MAL3-101–resistant RMS13-R cell line tolerated up to 30 μM MAL3-101 without loss of viability (Fig. 3*D*). Although RMS13-R cells activated the UPR appropriately after treatment with tunicamycin, CHOP induction was absent after MAL3-101 treatment, suggesting a resistance mechanism that prevented UPR activation (Fig. 3*E*). Whole-exome and transcriptome sequencing of RMS13-R cells failed to identify point mutations or copy number alterations in the HSP70 family members, PERK,

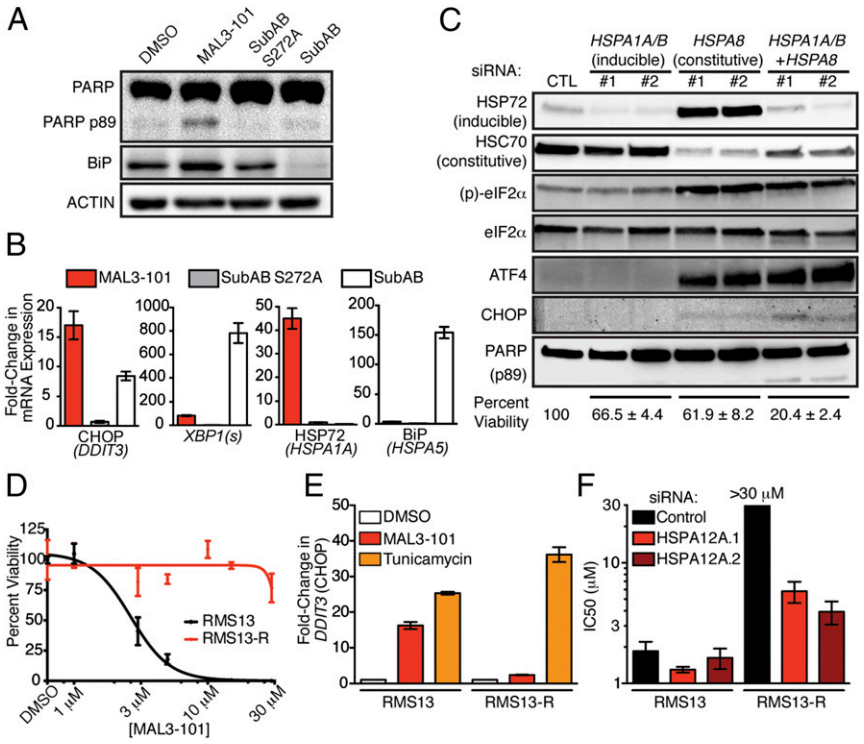

Fig. 3. Cytosolic HSP70 is required for RMS survival. (*A*) RMS13 cells were treated with DMSO, 10 μM MAL3-101, 125 ng/mL catalytically inactive subtilase cytotoxin (SubAB S272A), or 125 ng/mL active toxin (SubAB) for 6 h. Whole-cell lysates were used to detect PARP cleavage and BiP levels. Data shown represent three independent experiments. (*B*) RNA was extracted from RMS13 cells for qPCR as in *A*. Gene expression was normalized to *GAPDH*; data show mean ± SD ($n = 3$). (*C*) RMS13 cells were transfected with the indicated siRNAs for 72 h. Immunoblots confirmed knockdown and measured UPR activation. Data shown are representative of three independent experiments. Cell viability 120 h after transfection was measured by CellTiter-Glo and normalized to cells transfected with a control siRNA. Mean viability ± SEM from two siRNAs per gene are shown below blots ($n = 4$). (*D*) Dose–response of the isogenic RMS13-R cell line and parental RMS13 cells to MAL3-101. (*E*) RMS13 and RMS13-R cells were treated with 10 μM MAL3-101 or 5 μg/mL tunicamycin for 6 h. CHOP induction was measured by qPCR. Data are shown as mean fold-change ± SEM. (*F*) RMS13 and RMS13-R cells were transfected with siRNA as indicated and then were treated with MAL3-101; viability was measured using CellTiter-Glo. IC_{50} doses were calculated by nonlinear regression. IC_{50} for RMS13-R cells treated with control siRNA was not reached at the highest dose tested (30 μM). Mean IC_{50} (± SEM) from three independent replicates is shown.

eIF2α, or CHOP that could explain resistance but instead demonstrated significantly increased expression (but not mutation or genomic amplification) of a distinct cytosolic HSP70, heat shock protein family A member 12A (*HSPA12A*) (Table S3). We hypothesized that up-regulation of HSPA12A might promote resistance to MAL3-101 by increasing the abundance of a relevant drug target (i.e., an alternate cytosolic HSP70 protein). As predicted, silencing *HSPA12A* expression in RMS13-R cells resulted in partial restoration of MAL3-101 sensitivity (Fig. 3*F* and Fig. S4). Notably, these RMS13-R cells remained sensitive to a wide range of other therapeutic agents (Fig. S5) and did not up-regulate multidrug-resistance transporters (Fig. S6). These results strongly suggest that the mechanism underlying MAL3-101 resistance is inhibitor specific. Together, our data establish cytosolic HSP70 function as essential for RMS cell survival and show that MAL3-101 blocks cytosolic HSP70s to activate lethal PERK–eIF2α–CHOP signaling.

Conventional Chemotherapy Is Insufficient to Activate CHOP in RMS. We next sought to understand the clinical significance of CHOP expression in RMS patients. Interestingly, we found by analysis of an available mRNA profiling dataset that increased CHOP mRNA predicted decreased survival in RMS patients treated with conventional chemotherapy (Fig. 4*A*) (36). Consistent with this finding is the observation that genomic amplification of the 12q13-14 band containing CHOP (*DDIT3*), which likely increases CHOP mRNA expression, is associated with decreased event-free survival in fusion-positive RMS patients (37). This paradox—i.e., CHOP-dependent cancer cell death in cell lines but more aggressive cancer with high CHOP mRNA in clinical specimens—may be explained by the translational regulation of CHOP. Translation of full-length, functional CHOP proceeds from an internal ORF only upon phosphorylation of eIF2α (38), as occurs in response to HSP70 inhibition (Fig. 2*C*). Therefore, we hypothesized that conventional chemotherapy, unlike HSP70-targeted therapy, is insufficient to induce the translation of full-length CHOP in RMS cells despite high levels of CHOP mRNA. In support of this hypothesis, we found that the chemotherapeutic agents used in RMS patients were unable to induce eIF2α phosphorylation and CHOP protein expression substantially within the timeframe in which HSP70 inhibition was able to do so (Fig. 4*B*). Based on our collective findings, we posit that CHOP up-regulation predicts poor clinical response to chemotherapy because conventional treatment does not trigger the induction of functional CHOP protein that supports apoptosis in RMS cells. However, increased CHOP instead may identify a population of patients whose tumors are primed to undergo apoptosis in response to HSP70-targeted therapy, as predicted by our preclinical findings. Thus, CHOP may serve as a dual biomarker. This scenario is similar to human epidermal growth factor receptor 2 (*HER2*) amplification in patients with breast cancer, in whom *HER2* amplification predicts poor outcomes with conventional chemotherapy but superior outcomes with HER2-targeted therapy (39, 40). Consistent with this notion, CHOP promotes cell survival or death in a context-specific manner (24, 41). Overall, these findings reveal a potentially important dual and context-specific role for CHOP in RMS pathogenesis and identify

CHOP as a promising biomarker to guide the clinical development of cytosolic HSP70 inhibitors to treat RMS patients.

Discussion

We used a coordinated chemical–genetics strategy to reveal an unanticipated dependence of RMS cells on cytosolic HSP70, which we found acts to suppress UPR signaling from the ER and downstream CHOP engagement and apoptosis. This prosurvival role of HSP70 in RMS was independent of chaperone-mediated oncoprotein stabilization, supporting the application of our findings in both PAX3-FOXO1 fusion-positive and fusion-negative tumors. The central role of the CHOP transcription factor in this process highlights a potential biomarker for the efficacy of HSP70 inhibition. Our mechanistic insights also provide a new rationale for the therapeutic utility of HSP70-targeted therapy in RMS. Further development and optimization of MAL3-101 or a distinct cytosolic HSP70 inhibitor with favorable in vivo properties is necessary before preclinical testing and potential clinical translation of the therapeutic hypothesis arising from our work. Our findings provide strong motivation to pursue further pharmacologic development, because our data indicate that in vivo genetic studies would require successful knockdown of multiple constitutive and inducible forms of cytosolic HSP70 to represent pharmacologic modulation accurately. More broadly, our work provides a rationale for mapping proteostasis component coordination and function comprehensively in a cancer subtype-specific manner.

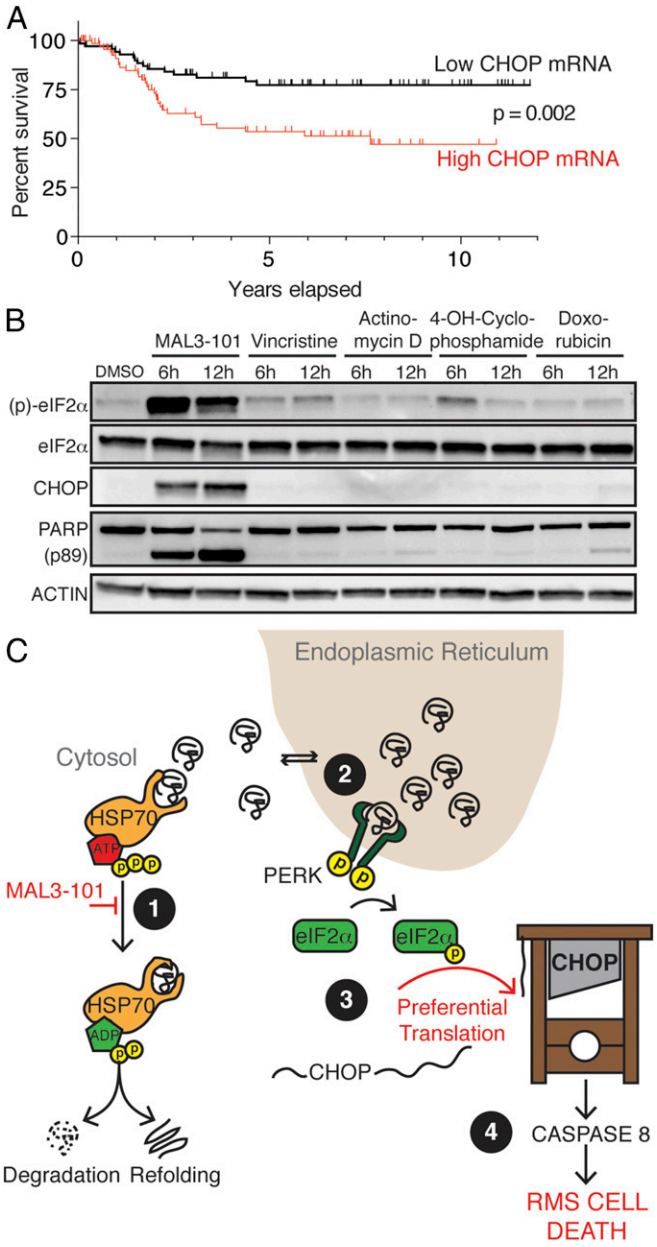

Fig. 4. Increased CHOP is a biomarker of poor survival in RMS patients after chemotherapy. (*A*) 120 RMS samples were divided by median expression of *DDIT3* (CHOP) from Affymetrix U133A microarrays (36). *P* value for survival was calculated by log-rank test. (*B*) Immunoblot of RMS13 cells treated with 10 μM MAL3-101, 5 nM vincristine, 5 nM actinomycin D, 5 μg/mL 4-hydroperoxy-cyclophosphamide, or 1 μM doxorubicin for the indicated times. (*C*) A model for HSP70 dependence in RMS. Cytosolic HSP70s degrade or refold unfolded proteins (1) that back up in the ER after MAL3-101 treatment (2), thereby activating PERK to phosphorylate eIF2α (3). When CHOP mRNA is abundant, eIF2α phosphorylation leads to rapid translation of CHOP protein (4), activating a lethal program culminating in caspase 8 activation.

Our observation that inhibition of cytosolic HSP70 in RMS activates the UPR uncovers a connection between the function of this chaperone and the UPR. Although modest inhibition of the ER HSP70 chaperone BiP may contribute to the lethal effects of MAL3-101, our genetic data argue that loss of the cytosolic HSP70 chaperones is sufficient to activate UPR-mediated cell death. Precedent for such a connection has been established previously. For example, HSP70 inhibition might lead to the formation of cytosolic aggregates, and previous work demonstrated that poly-glutamine aggregates directly interfere with components of the ER-associated degradation (ERAD) pathway, which in turn induces the UPR (42). We also showed previously that perturbing cytosolic HSP70 disables ERAD and triggers the UPR, particularly when membrane-integrated, misfolded ER proteins must be cleared (43). Future studies will help elucidate the potential role of ERAD in connecting cytosolic HSP70 function to the UPR and CHOP induction in RMS cells.

We propose a model (Fig. 4*C*) whereby RMS-specific wiring of the proteostasis network links cytosolic HSP70 to CHOP through the UPR and is responsible for HSP70 inhibitor sensitivity in this disease. The specific factors underlying the dependence of RMS cells upon cytosolic HSP70 function remain to be identified. We propose two possibilities that are not necessarily mutually exclusive. First, the protein-translation program of RMS cells might include a significant number of HSP70 (and more specifically, ERAD) clients that accumulate upon cytosolic HSP70 inhibition, triggering the lethal UPR-inducible axis we uncovered. Future proteomic approaches may reveal clients whose clearance from the ER is blocked by HSP70 inhibition. Second, stress-sensing pathways and effectors (such as CHOP) in RMS cells might be hypersensitive to perturbations in proteostasis. Supporting this model is prior work implicating eIF2α–ATF4–CHOP signaling both in myogenesis, where CHOP may prevent terminal differentiation (44), and in sarcomagenesis (45). The duration of UPR signaling also has been linked to a bias toward apoptotic rather than adaptive (prosurvival) output (46), suggesting a mechanism by which myoblasts use CHOP in a regulated manner downstream of UPR engagement as a survival factor to establish pathogenicity and initiate RMS. However, sustained CHOP protein up-regulation in established RMS cells may then instead trigger an alternative fate, i.e., apoptosis in response to HSP70 inhibition. Because chemotherapy is insufficient to induce CHOP, HSP70 inhibition exploits an unanticipated vulnerability in this cancer.

In the era of precision medicine, the identification of driver mutations has yielded significant advances in the treatment of certain cancers (47). Unfortunately, progress in many other cancers, including RMS, has lagged. Our discovery of HSP70 dependence in RMS demonstrates that using chemical–genetic approaches to decipher and target the key and specific supportive

networks that particular cancer cell types use to compensate for the stresses of oncogenic transformation—the so-called "non-oncogene dependence networks"—can provide novel, biomarker-driven therapeutic strategies to improve survival in patients in whom direct oncoprotein inhibition is not currently possible (48).

Methods

Cell lines are described in *SI Methods*. AJSR1 cells were grown from a marrow aspirate from the University of California, San Francisco (UCSF) Pediatric Tissue Bank and PAX3-FOXO1 expression confirmed by RT-PCR (Fig. S7). Informed consent was obtained under a protocol reviewed by the UCSF Institutional Review Board (IRB Approval 11-05192). MAL3-101 was dissolved in DMSO to 20 mM and stored at −80 °C. CellTiter-Glo (Promega) was used per the manufacturer's instructions to measure cell viability. Antibodies for immunoblots are listed in *SI Methods*. RNA was extracted using RNeasy kits (Qiagen); quantitative PCR (qPCR) was performed as described (49) using primers as listed (Table S3); and transcriptome analysis was performed on an Illumina HiSEq. 2500 platform. Reads were aligned to hg19 using RSEM (50), and expression was calculated with DESeq. (51). The RMS13-R line was created by culturing RMS13 cells in escalating doses of MAL3-101 for 2 mo. Further details are given in *SI Methods*.

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