Engineered protein disaggregases mitigate toxicity of aberrant prion-like fusion proteins underlying sarcoma

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Edited by Paul E. Fraser

FUS and EWSR1 are RNA-binding proteins with prion-like domains (PrLDs) that aggregate in amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD). The FUS and EWSR1 genes are also prone to chromosomal translocation events, which result in aberrant fusion proteins between portions of the PrLDs of FUS and EWSR1 and the transcription factors CHOP and FLI. The resulting fusion proteins, FUS-CHOP and EWS-FLI, drive aberrant transcriptional programs that underpin liposarcoma and Ewing's sarcoma, respectively. The translocated PrLDs alter the expression profiles of these proteins and promote their phase separation and aggregation. Here, we report the development of yeast models of FUS-CHOP and EWS-FLI toxicity and aggregation. These models recapitulated several salient features of sarcoma patient cells harboring the FUS-CHOP and EWS-FLI translocations. To reverse FUS and EWSR1 aggregation, we have explored Hsp104, a hexameric AAA+ protein disaggregase from yeast. Previously, we engineered potentiated Hsp104 variants to suppress the proteotoxicity, aggregation, and mislocalization of FUS and other proteins that aggregate in ALS/FTD and Parkinson's disease. Potentiated Hsp104 variants that robustly suppressed FUS toxicity and aggregation also suppressed the toxicity and aggregation of FUS-CHOP and EWS-FLI. We suggest that these new yeast models are powerful platforms for screening for modulators of FUS-CHOP and EWS-FLI phase separation. Moreover, Hsp104 variants might be employed to combat the toxicity and phase separation of aberrant fusion proteins involved in sarcoma.

Chromosomal translocation events underpin sarcoma (1). In liposarcoma, the N-terminal portion of FUS (2) becomes aberrantly fused to the transcription factor CHOP (1). In Ewing's sarcoma, the second most common pediatric bone cancer, a chromosomal translocation event inappropriately fuses the N-terminal region of EWSR1 to the transcription factor FLI (1). The EWS-FLI translocation can occur in multiple frames, leading to the formation of different translocation products (1). Due to their fusion to portions of FUS and EWSR1, the transcription factors become differentially regulated and elicit aberrant transcriptional programs (1). For instance, FLI is expressed only under specific conditions, whereas EWSR1 is constitutively expressed (1). Fusion of FLI to EWSR1 results in constitutive expression of FLI in the form of the EWS-FLI fusion. Both FUS and EWSR1 harbor prion-like domains (PrLDs), and large portions of these PrLDs are retained in the translocated products (1, 2). PrLDs are found in ~240 human proteins and resemble yeast prion domains in their aggregation propensity and precise low-complexity amino acid composition, which is enriched for uncharged polar residues and glycine (3–6). The PrLDs of FUS and EWSR1 drive the aggregation and toxicity of full-length FUS and EWSR1, which aggregate in subsets of patients with amyotrophic lateral sclerosis (ALS) or frontotemporal dementia (FTD) (7–13). Thus, in addition to altered expression profiles, the PrLDs of FUS and EWSR1 may dramatically alter the biochemical properties of CHOP and FLI (2, 12). In fact, the PrLD of EWS-FLI promotes aberrant phase separation events, which lead to the inappropriate recruitment of chromatin-remodeling factors, activating the deleterious transcriptional events of Ewing's sarcoma (2). Thus, agents that antagonize these aberrant phase separation events could be therapeutic for sarcoma (2, 14–16).

To study human disease in a highly genetically tractable model system, we and others have demonstrated that diverse protein-misfolding disorders can be modeled in the budding yeast, Saccharomyces cerevisiae (7–9, 17–24). Excellent models have been developed for studying FUS and EWSR1 misfolding (in ALS/FTD) as well as α-synuclein (in Parkinson's disease) and TDP-43 (in ALS/FTD) among others (7–9, 17–24). Yeast models recapitulate many of the molecular phenotypes observed in patients with these respective disorders, and they are also highly genetically tractable platforms, which enable rapid screening for genetic and small-molecule modifiers (7, 17–19, 21, 23–26). For instance, the RNA-binding protein FUS shuttles to the nucleus to fulfill its roles in regulating RNA homeostasis (10). However, in ALS/FTD patients and in yeast models of FUS proteinopathy, FUS is toxic and aggregates in
the cytoplasm (7, 10, 11, 21). This yeast system has been used to identify potent genetic modifiers of FUS aggregation and toxicity (7, 21, 27, 28). Here, we establish yeast models for studying the aggregation of FUS-CHOP and EWS-FLI. Additionally, we assessed whether protein disaggregases that dissolve FUS aggregates might also be capable of dispersing altered FUS-CHOP and EWS-FLI phases (27, 29, 30).

Prions are not invariably toxic or harmful (31–34). In S. cerevisiae, prions are harnessed for adaptive purposes, and so they are tightly regulated (35, 36). The AAA+ protein Hsp104 constructs and deconstructs yeast prions and solubilizes proteins that accumulate after environmental stress (37–51). We hypothesized that the shared cross-β structure of amyloids might enable a protein that naturally regulates amyloid in yeast to be active against similarly structured proteins that underpin human disease (39, 52, 53). However, Hsp104 has somewhat limited ability to disaggregate proteins that aggregate in human disease (27, 54, 55). Thus, we have used protein engineering to potentiate Hsp104 (56). We have found that potentiated Hsp104 variants suppress the misfolding and toxicity of diverse proteins that form amyloid or harbor prion-like domains (27, 29, 57–61). Enhanced Hsp104 disaggregases suppress the toxicity of TDP-43, FUS, and α-synuclein as well as disease-associated mutants of these proteins, and they suppress toxicity under conditions where WT Hsp104 is ineffective (27, 29, 47, 58 –60). Although each of the potentiated Hsp104 variants was isolated from a screen against a single disease substrate, the majority of the variants isolated thus far rescue the toxicity of TDP-43, FUS, and α-synuclein (27). Certain variants also suppress dopaminergic neurodegeneration in a Caenorhabditis elegans model of Parkinson’s disease (27). They also reverse FUS aggregation in fibroblasts while restoring proper localization of FUS-associated RNA (30). Surprisingly, although these variants rescue diverse substrates harboring PrLDs, they do not suppress the toxicity and aggregation of EWSR1, which has a domain architecture strikingly similar to that of FUS (29).

Here, we explored the effects of FUS-CHOP and EWS-FLI overexpression in yeast and found that they are toxic and aggregate in patterns resembling those in sarcoma patients. We further demonstrate that FUS-CHOP undergoes aberrant phase separation, adopting a gel- or solid-like state. We also demonstrate that just as the potentiated Hsp104 variants counter the toxicity of FUS, they can also suppress the toxicity of the FUS-CHOP and EWS-FLI translocations. We therefore propose that these yeast model systems might be useful platforms for studying the underpinnings of sarcoma as well as for screening for modifiers of these disorders. Moreover, we suggest that engineered protein disaggregases might be employed to combat aberrant phenotypes driven by prion-like fusion proteins involved in sarcoma and other cancers.

Results

**FUS-CHOP is toxic and aggregates when expressed in yeast**

FUS is a nuclear RNA-binding protein with a PrLD that forms cytoplasmic aggregates in the degenerating neurons of ALS/FTD patients (10). FUS forms toxic cytoplasmic aggregates in yeast just as it does in the degenerating neurons of ALS/FTD patients (7, 21). Therefore, we established a similar model for studying the expression of the FUS-CHOP translocation, which is implicated in liposarcoma (Fig. 1A) (1).

To assess FUS-CHOP toxicity and probe the domain requirements that might drive this toxicity, we expressed FUS-CHOP and several related constructs in yeast (Fig. 1A). We used the 413GAL plasmid to drive galactose-inducible expression of these genes, and we expressed 413GAL (empty vector), 413GAL-FUS, 413GAL-FUS-CHOP, 413GAL-FUS(1–266), and 413GAL-FUS-CHOP. FUS-CHOP overexpression was highly toxic and slightly more toxic than full-length FUS (Fig. 1B). The FUS(1–266) truncation was not toxic (Fig. 1B), corroborating studies demonstrating that the PrLD, RNA-recognition motif (RRM), and first arginine/glycine-rich (RRG) domain are required for FUS toxicity in yeast (7, 21). Furthermore, overexpression of CHOP alone is only very slightly toxic (Fig. 1B). These results demonstrate that both CHOP and the PrLD of FUS are required to elicit severe FUS-CHOP toxicity. We confirmed that each of the proteins was expressed by using anti-FUS and anti-CHOP antibodies (Fig. 1C). Each of the proteins was expressed, and FUS(1–266) was expressed at a lower level.

Using fluorescence microscopy, we established that FUS-CHOP-GFP forms nuclear foci in yeast (Fig. 1D) just as it does in myxoid liposarcoma cells (62, 63). FUS(1–266)-GFP displays more diffuse fluorescence than FUS-CHOP-GFP with some nuclear foci (Fig. 1D). Furthermore, CHOP-GFP accumulates diffusely in the nucleus, correlating with its role as a transcription factor. To confirm the fusion to GFP did not substantially alter the toxicity of these proteins, we repeated spotting assays for the GFP-tagged constructs, and results were similar to those for the untagged constructs (Fig. 1, B and E). Thus, the nonaggregating CHOP is minimally toxic, and aggregation of FUS(1–266) is not toxic (7). Because FUS(1–266) does not bind nucleic acid (64), these findings suggest that aggregation of the PrLD alone is not sufficient for toxicity. Rather, these findings suggest that constructs must aggregate and be capable of engaging nucleic acid to confer toxicity as with FUS-CHOP and full-length FUS (7, 65, 66). Likewise, TDP-43 must aggregate and be able to engage RNA to elicit toxicity in yeast and other model systems (19, 20, 67–69).

In summary, FUS-CHOP aggregates in the nucleus and is toxic in yeast, recapitulating cancer cell phenotypes observed in liposarcoma patients (62, 63). Our results suggest that yeast is an excellent model system for studying FUS-CHOP aggregation and toxicity and that this yeast model might be a suitable platform for unearthing small-molecule or genetic modulators of FUS-CHOP aggregation and toxicity.

**EWS-FLI is toxic and aggregates when expressed in yeast**

Like FUS, EWSR1 is a RNA-binding protein with a PrLD, and its aggregation is implicated in a subset of ALS and FTD patients (8, 70, 71). Thus, we also assessed the toxicity of EWS-FLI in yeast and performed similar domain-mapping experiments (Fig. 2A). Here, we expressed EWS-FLI1 and EWS-FLI3 in yeast from the galactose-inducible 413GAL plasmid along with controls 413GAL (empty vector), 413GAL-EWSR1, 423GAL-EWSR1, 413GAL-EWS(1–264), 413GAL-EWS(1–266), 413GAL-EWS(267–413), and 413GAL-EWS(1–523). Expression of EWS-FLI in yeast just as it does in the degenerating neurons of ALS/FTD patients (7, 21). Therefore, we established a similar model for studying the expression of the EWS-FLI translocation, which is implicated in liposarcoma (Fig. 1A) (1).
EWS-FLI1 and EWS-FLI3 overexpression was highly toxic and far more toxic than full-length EWSR1 when expressed at similar levels (Fig. 2B and C). We also expressed EWSR1 from a 2μ plasmid to increase EWSR1 expression. Even at these higher expression levels (Fig. 2C), the EWS-FLI constructs are more toxic than EWSR1 (Fig. 2B). Expression of neither EWS(1–264) nor EWS(1–347) was toxic, and expression of FLI was only slightly toxic. Thus, the fusion of a portion of the EWSR1 PrLD to FLI confers a synergistic enhancement of toxicity in yeast. We confirmed that each of these proteins was expressed by using anti-EWS and anti-FLI antibodies (Fig. 2C). Although each of the proteins was expressed, detection of the EWS(1–264) truncation was weaker (Fig. 2C).

Next, we assessed the aggregation of these constructs in yeast. We first constructed GFP-tagged versions of the strains and confirmed that their toxicity was similar to that of the untagged constructs (Fig. 2B and D). We next confirmed that EWS-FLI1-GFP and EWS-FLI3-GFP aggregate in yeast and found that these foci accumulate in the nucleus (Fig. 2E). As with FUS(1–266), expression of EWS(1–264) or EWS(1–347) yielded cells with more diffuse fluorescence than EWS-FLI1 and some nuclear foci, although again fewer than for the EWS-FLI constructs (Fig. 2E). Also, as with FUS-CHOP, these constructs consisting primarily of the EWSR1 PrLD yielded larger foci than for the full-length fusion (Fig. 2E). Finally, consistent with its role as a transcription factor, localization of FLI alone was restricted primarily to diffuse staining inside the nucleus (Fig. 2E). These data suggest that, as with FUS-CHOP, the PrLD of EWSR1 must be appended to a nucleic acid–binding domain to connect aggregation and toxicity. Thus, FLI does not aggregate and is not toxic, and the PrLD portions of EWSR1 aggregate but are not toxic. By contrast, appending portions of the EWSR1 PrLD to either an RRM plus RGG domains as in EWSR1 or to a DNA-binding domain as in EWS-FLI yields proteins that aggregate and are toxic.

In summary, like FUS-CHOP, EWS-FLI1 and EWS-FLI3 aggregate and are toxic in yeast, recapitulating cellular phenotypes found in Ewing’s sarcoma patients (1, 2). Our results demonstrate that yeast is an excellent model system for studying FUS-CHOP and EWS-FLI aggregation and toxicity and that these models could be excellent platforms for discovering
small-molecule or genetic modifiers of the toxicity of these translocations.

**FUS-CHOP protein is immobile within nuclear inclusions**

It has been shown that the translocated PrLD of EWSR1 can elicit phase separation events that can disrupt transcriptional programs and lead to cancer (2). We assessed whether these same aberrant phase transitions applied to our yeast models and more broadly to FUS-CHOP. Using super-resolution imaging, we first visualized cells expressing FUS-CHOP-GFP, EWS-FLI1-GFP, or EWS-FLI3-GFP. Cells expressing FUS-CHOP-GFP were characterized by foci ~600 nm in diameter, whereas those for EWS-FLI measured less than 300 nm in diameter (Fig. 3A). Furthermore, the EWS-FLI foci were highly dynamic, moving rapidly throughout the nucleus. Although the FUS-CHOP inclusions also were prone to movement, this movement was slower than for EWS-FLI. As a result, we were only able to characterize the material properties of the FUS-CHOP inclusions using fluorescence recovery after photobleaching (FRAP). We employed FRAP to determine whether these inclusions displayed internal rearrangements characteristic of a liquid or a solid. Here, we photobleached a small region of the nuclear foci and tracked the recovery of fluorescence intensity within bleached and unbleached regions of interest (ROIs) by confocal microscopy (Fig. 3B). On following the inclusions for 90 s, we noted minimal recovery of fluorescence. Materials in the liquid state would be expected to adopt a spherical shape (72). However, the FUS-CHOP inclusions do not appear to be spherical, suggesting that FUS-CHOP adopts a gel- or solid-like state in yeast (Fig. 3B). For comparison, we also bleached cells expressing GFP with no fused protein. Recovery of FUS-CHOP-GFP reached only ~40% of the prebleached fluorescence intensity at most, whereas recovery of GFP exceeded 60% and was more rapid (Fig. 3C). Thus, we conclude that FUS-CHOP phase separates to a gel- or solid-like state.

*Enhanced protein disaggregases to counter sarcoma*

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**Figure 2. EWS-FLI is toxic and forms nuclear foci in yeast.**

- **A**, domain architecture of EWSR1, EWS-FLI1, EWS-FLI3, and the corresponding truncations. **DBD**, DNA-binding domain; **AD**, activation domain.
- **B**, W303Δhis104 yeast was transformed with galactose-inducible EWSR1, EWS-FLI1, EWS-FLI3, or the indicated truncation. The strains were serially diluted 5-fold and spotted on glucose (off) or galactose (on) media. **C**, strains from **B** were induced for 5 h, lysed, and immunoblotted. PGK1 serves as a loading control. **D**, spotting of the GFP-tagged constructs was performed as in **B**. **E**, fluorescence microscopy of cells expressing EWS-FLI1-GFP and EWS-FLI3-GFP and the truncations stained with Hoechst dye to visualize nuclei (blue). **Scale bar**, 5 μm.
Potentiated Hsp104 variants suppress FUS-CHOP toxicity and aggregation

We have previously demonstrated that engineered variants of the yeast AAA+/H11001 protein Hsp104 can suppress the aggregation and toxicity of FUS, TDP-43, and α-synuclein (27, 29, 58). Hsp104 is a protein disaggregase that translocates these substrates through its central pore and, through cycles of ATP hydrolysis, dissolves these kinetically trapped aggregates such that solubilized proteins can then refold to the native state (39, 52, 53, 73, 74). We hypothesize that, as for EWS-FLI (2), the PrLD of FUS-CHOP drives FUS-CHOP aggregation in addition to altering its transcriptional program (62, 63). Thus, development of a FUS-CHOP disaggregase might be a useful agent to counter aberrantly functioning FUS-CHOP.

We were curious whether the same Hsp104 variants that can dissolve FUS aggregates might also rescue FUS-CHOP toxicity, considering that the PrLD of FUS is conserved in the FUS-CHOP translocation (1, 27, 29). Hsp104 harbors a nuclear localization sequence and localizes to the cytoplasm and nucleus in yeast (75). Thus, Hsp104 could antagonize formation of nuclear FUS-CHOP aggregates. We coexpressed FUS-CHOP with Hsp104 and a series of potentiated variants, Hsp104A503V, Hsp104A503S, Hsp104 A503G, Hsp104 V426L, Hsp104 A437W, Hsp104Y507C, Hsp104 N539K, and Hsp104 DPLF-A503V (27).

Hsp104 is inactive against FUS in yeast, and we found that Hsp104 is similarly inactive against FUS-CHOP (Fig. 4A). However, each of the potentiated Hsp104 variants tested potently suppressed FUS-CHOP toxicity, restoring growth to nearly the levels of vector alone (Fig. 4A, top lane, 413GAL + 416GAL). We assessed expression levels by immunoblotting and confirmed that this toxicity suppression is not due to decreased FUS-CHOP expression (Fig. 4B). As we have seen in other studies (27, 29, 58), the Hsp104 variants are expressed at lower levels than Hsp104, suggesting that they are more active at lower concentrations.

We next assessed the effects of the potentiated Hsp104 variants on the FUS-CHOP nuclear foci. Here, we coexpressed FUS-CHOP-GFP with Hsp104 and two of the potentiated variants, Hsp104A503V and Hsp104A503S. Although Hsp104 has no apparent effect on the FUS-CHOP-GFP foci, cells expressing either Hsp104A503V or Hsp104A503S show a reduced number and size of nuclear foci (Fig. 4, C and D). We confirmed that the Hsp104 variants only subtly decrease expression levels of FUS-CHOP-GFP (Fig. 4E). Because we still observed some foci, we tested the rescue of the potentiated variants against FUS-CHOP-GFP (Fig. 4F). We found that FUS-CHOP-GFP is less toxic than untagged FUS-CHOP, and so the rescue of toxicity is more modest than for the untagged constructs. Here, Hsp104A503S was more effective in rescuing toxicity than Hsp104A503V (Fig. 4F). Collectively, these findings establish that enhanced Hsp104 variants antagonize FUS-CHOP toxicity and phase separation.

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Potentiated Hsp104 variants suppress EWS-FLI toxicity and aggregation

We have previously shown that the substrate repertoire of the potentiated Hsp104 variants is broad (27, 29). Thus, we hypothesized that, given the similar domain architecture of EWSR1 and FUS, Hsp104 variants that disaggregate FUS would also disaggregate EWSR1. Surprisingly, variants active against

Figure 3. FUS-CHOP inclusions display gel- or solid-like properties. A, FUS-CHOP, EWS-FLI1, and EWS-FLI3 form nuclear foci in yeast, and FUS-CHOP foci were generally substantially larger than EWS-FLI foci. B, FRAP of FUS-CHOP-GFP shows that the nuclear foci are immobile and do not recover after 90 s. Also, the inclusions do not appear spherical, consistent with more gel- or solid-like properties. Arrows indicate the ROI that was photobleached. C, FRAP experiments were performed for FUS-CHOP-GFP and a diffuse GFP control. FUS-CHOP shows a slower rate of return than GFP. Scale bars in A and B are 500 nm. Curves in C show data points (dark blue and red) ± S.E. (light blue and red), n = 10 cells per condition.
FUS do not rescue EWSR1 toxicity or aggregation, and some variants even enhance EWSR1 toxicity (29). Therefore, we were curious to test whether these same variants might rescue EWS-FLI toxicity. We coexpressed EWS-FLI1 and EWS-FLI3 with Hsp104 and the same series of potentiated variants tested against FUS-CHOP. Hsp104 does not suppress EWSR1 toxicity in yeast, and we found that Hsp104 does not suppress EWS-FLI1 or EWS-FLI3 toxicity either (Fig. 5, A and C). We found that, unlike with FUS-CHOP, only certain Hsp104 variants rescue EWS-FLI1 toxicity: Hsp104A503V, Hsp104A503G, Hsp104A437W, Hsp104N539K, and Hsp104DPLF-A503V robustly rescue EWS-FLI1 toxicity. By contrast, Hsp104A503V confers a more modest rescue of EWS-FLI1 toxicity. We confirmed that this rescue was not simply due to decreased EWS-FLI1 expression levels (Fig. 5B). Hsp104A503S, Hsp104A503G, Hsp104N539K, and Hsp104DPLF-A503V confer a strong rescue of EWS-FLI3 toxicity, whereas Hsp104V426L and Hsp104A437W confer a more modest rescue (Fig. 5C). As with EWS-FLI1, the potentiated variants rescue EWS-FLI3 toxicity without substantially modifying EWS-FLI3 expression levels (Fig. 5D). Like we have seen with FUS-CHOP, the Hsp104 variants are expressed at lower levels than Hsp104, indicating that they are active even when expressed at lower concentrations. Although Hsp104A503S and Hsp104A503V do not rescue EWSR1 toxicity (29). These results suggest that translocation of the EWSR1 PrLD to

Figure 4. Potentiated Hsp104 variants suppress FUS-CHOP toxicity and aggregation. A, W303Δhsp104 yeast was sequentially transformed with FUS-CHOP and Hsp104 or the indicated variants. The strains were serially diluted 5-fold and spotted on glucose (off) or galactose (on) media. B, strains from A were induced for 5 h, lysed, and immunoblotted. C, W303Δhsp104 yeast was sequentially transformed with FUS-CHOP-GFP and Hsp104 or the indicated variants. The strains were induced for 5 h, stained with Hoechst dye, and imaged. Representative images are shown. Scale bar, 5 μm. D, quantification of microscopy experiments shown in C. Error bars represent S.E.M. E, strains from C were induced for 5 h, lysed, and immunoblotted. F, strains from C were serially diluted 5-fold and spotted on glucose and galactose media.

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A

413 GAL + 416 GAL
416 GAL
416 GAL Hsp104WT
416 GAL Hsp104:A503V
416 GAL Hsp104:A503S
416 GAL Hsp104:A503G
416 GAL Hsp104:V426L
416 GAL Hsp104:A437W
416 GAL Hsp104:Y507C
416 GAL Hsp104:N539K
416 GAL Hsp104:DPLF-A503V

Glucose (off) Galactose (on)

B

EWS-FLI1 +

Vector
Hsp104WT
A503V
A503S
A437V
Y426L
Y507C
N539K
DPLF-A503V
No induction

α-Hsp104
α-EWS
α-PGK

100 kD
75 kD
37 kD

C

416 GAL
416 GAL Hsp104WT
416 GAL Hsp104:A503V
416 GAL Hsp104:A503S
416 GAL Hsp104:A503G
416 GAL Hsp104:V426L
416 GAL Hsp104:A437W
416 GAL Hsp104:Y507C
416 GAL Hsp104:N539K
416 GAL Hsp104:DPLF-A503V

Glucose (off) Galactose (on)

D

EWS-FLI3 +

Vector
Hsp104WT
A503V
A503S
A437V
Y426L
Y507C
N539K
DPLF-A503V
No induction

α-Hsp104
α-EWS
α-PGK

100 kD
75 kD
37 kD

E

Vector
Hsp104WT
Hsp104:A503V
Hsp104:A503S
Hsp104:A503G
Hsp104:V426L
Hsp104:A437W
Hsp104:Y507C
Hsp104:N539K
Hsp104:DPLF-A503V

Glucose (off) Galactose (on)

F

EWS-FLI1-GFP EWS-FLI3-GFP

+ Vector + Hsp104WT + A503V + A503S + Hsp104:V426L + A437V + Y507C + N539K + DPLF-A503V

α-Hsp104
α-GFP
α-PGK

100 kD
37 kD

G

EWS-FLI1-GFP EWS-FLI3-GFP

+ Vector
+ Hsp104WT
+ Hsp104:A503V
+ Hsp104:A503S

GFP
Hoechst
Merge

% of Cells with Fold
FLI yields a protein with physical properties distinct from EWSR1, allowing for detoxifying remodeling by the potentiated Hsp104 variants.

We next assessed the effects of the potentiated Hsp104 variants on the EWS-FLI nuclear foci. Here, we coexpressed EWS-FLI1-GFP or EWS-FLI3-GFP with Hsp104 or two of the potentiated variants that conferred a mild (Hsp104<sub>A<sup>503V</sup></sub>) and more moderate (Hsp104<sub>A<sup>503S</sup></sub>) rescue of toxicity. We first restested these variants against the EWS-FLI-GFP fusions to confirm they still rescue toxicity (Fig. 5E). EWS-FLI1-GFP and EWS-FLI3-GFP are both less toxic than their untagged versions, and so the potentiated Hsp104 variants rescue this toxicity more robustly. We then confirmed expression via immunoblotting and found that the Hsp104 variants decrease the expression of EWS-FLI-GFP more than they do for EWS-FLI (Fig. 5B, D and F). Using fluorescence microscopy, we found that, although Hsp104 has no effect on the EWS-FLI foci, cells expressing either Hsp104<sub>A<sup>503V</sup></sub> or Hsp104<sub>A<sup>503S</sup></sub> reduced nuclear foci (Fig. 5G). Collectively, our findings suggest that enhanced Hsp104 variants antagonize EWS-FLI toxicity and phase separation.

**Discussion**

Here, we have established yeast models to investigate the misfolding of FUS-CHOP and EWS-FLI translocations that underpin sarcoma. We found that fusion of FUS and EWS to CHOP and FLI, respectively, drives a synergistic enhancement in toxicity of the resulting fusions. As in sarcoma patients, FUS-CHOP and EWS-FLI expression in yeast leads to the formation of multiple nuclear foci. We found that FUS-CHOP foci display properties characteristic of a gel or solid rather than a liquid. Aggregation and nucleic acid–binding capability were important for toxicity as CHOP or FLI did not aggregate and were not toxic, and portions of the PrLDs of FUS or EWSR1 (which do not bind RNA) aggregated but were not toxic. In this regard, FUS-CHOP and EWS-FLI toxicity resembles FUS and TDP-43 toxicity in yeast where nucleic acid binding couples aggregation to toxicity (7, 19–21).

We also have demonstrated that potentiated Hsp104 variants that suppress FUS toxicity also robustly suppress the toxicity of FUS-CHOP and EWS-FLI. These Hsp104 variants also decrease the accumulation of nuclear foci of FUS-CHOP and EWS-FLI. It is important to note that, although these Hsp104 variants cannot counter the toxicity of EWSR1 (29), they do counter the toxicity of EWS-FLI. Thus, the EWS-FLI translocation yields a protein with a distinct structure, which enables the Hsp104 variants to remodel EWS-FLI even though they do not remodel EWSR1.

Recently, it has been demonstrated that the PrLD of EWS-FLI drives phase separation and that this phase separation activates the transcriptional events of Ewing’s sarcoma (2, 12). Hsp104 has been shown to regulate phase transitions in yeast (41, 48, 76). Thus, it will be interesting to determine whether the potentiated variants can also antagonize aberrant phase transitions of EWS-FLI or FUS-CHOP in sarcoma patient cells.

It has been demonstrated that many complex diseases can be modeled in yeast (7, 20–22, 77–79), and we now demonstrate that the aberrant phase transitions of FUS-CHOP and EWS-FLI that underlie sarcoma can also be modeled in yeast. We anticipate that these new model systems will serve as robust platforms to enable rapid genome-wide and small-molecule screens in yeast to identify additional modifiers of FUS-CHOP and EWS-FLI aggregation and toxicity.

We have demonstrated that Hsp104, which regulates yeast prions (80), can be potentiated to counter the toxicity of FUS, TDP-43, a-synuclein, and now FUS-CHOP and EWS-FLI as well (27, 29). It is intriguing that the specificity Hsp104 has for yeast prions carries over to substrates that form amyloid or prion-like structures in humans even though yeast Hsp104 has not encountered these exact substrates throughout evolution. In the future, it will be interesting to assess whether these enhanced disaggregases can also block the aberrant transcriptional programs driven by FUS-CHOP and EWS-FLI in sarcoma.

**Experimental procedures**

All yeast were WT W303a <del>hsp104</del> (MATa, can1–100, his3–11,15, leu2–3,112, trp1–1, ura3–1, ade2–1) (81). Yeast were grown in rich medium (yeast extract, peptone, dextrose (YPD)) or in synthetic media lacking the appropriate amino acids. Media were supplemented with 2% glucose, raffinose, or galactose. Vectors encoding FUS-CHOP and EWS-FLI were obtained from Addgene and Jeffrey Toretsky, respectively. The FUS and EWSR1 plasmids were described previously (27, 29). Vectors were cloned from these plasmids into pDONR 221 and then transferred to pAG413GAL via Gateway cloning. pAG416GAL-Hsp104 and the potentiated variants were generated previously (27). All truncated constructs were generated using QuikChange site-directed mutagenesis (Agilent), and all constructs were confirmed by DNA sequencing.

Yeast were transformed according to standard protocols using PEG and lithium acetate (82). Yeast strains were constructed by first transforming the appropriate pAG413GAL plasmid. Plates were then scraped, and cultures were inoculated and subsequently transformed with the pAG416GAL-Hsp104 plasmids. For the spotting assays, yeast were grown to saturation overnight in raffinose-supplemented dropout media at 30 °C. Cultures were serially diluted 5-fold and spotted in duplicate onto synthetic dropout media containing glucose or galactose. Plates were analyzed after growth for 2–3 days at 30 °C.

For immunoblotting, yeast were grown overnight to saturation in raffinose. They were then diluted to an A<sub>600 nm</sub> of 0.3 in galactose-containing medium and induced for 5 h. Cultures were normalized to an A<sub>600 nm</sub> of 0.6, 3 ml of cells were harvested and treated in 0.1 M NaOH for 5 min at room temperature, and cell
pullets were then resuspended into 1 × SDS sample buffer and boiled for 10 min. Lysates were cleared by centrifugation at 14,000 rpm for 2 min, then separated by SDS-PAGE (4–20% gradient; Bio-Rad), and transferred to a polyvinylidene difluoride membrane. Membranes were blocked in LI-COR Biosciences Odyssey PBS blocking buffer for 2 h at room temperature. Primary antibody incubations were performed at 4 °C overnight. Antibodies used included anti-FUS polyclonal (Bethyl Laboratories), anti-Hsp104 polyclonal (Enzo Life Sciences), anti-3-phosphoglycerate kinase (PGK) monoclonal (Invitrogen), anti-CHOP (Abcam), anti-EWS (Santa Cruz Biotechnology), and anti-FI1 (Abcam). Blots were processed using a LI-COR Biosciences Odyssey Fc imaging system.

For fluorescence microscopy, FUS-CHOP, EWS-FLI1, and EWS-FLI3 were imaged by appending a C-terminal GFP tag using Gateway cloning into pAG303GAL-ccdB-GFP. The pAG303GAL-FUS-CHOP-GFP, pAG303GAL-EWS-FLI1-GFP, and pAG303GAL-EWS-FLI3-GFP were then linearized and transformed as described above. Single colonies were selected, and yeast were grown and processed for microscopy as for immunoblotting. To assess the effects of Hsp104 on FUS-CHOP and EWS-FLI microtubular foci, these strains were then sequentially transformed with pAG416GAL, pAG416GAL-CHOP and EWS-FLI nuclear foci, these strains were then selected, and yeast were grown and processed for microscopy as for immunoblotting. To assess the effects of Hsp104 on FUS-CHOP and EWS-FLI microtubular foci, these strains were then sequentially transformed with pAG416GAL, pAG416GAL-Hsp104, or variants of Hsp104. For imaging the truncation variants, the pAG413GAL-ccdB-GFP plasmid was used in place of pAG303GAL-ccdB-GFP. After 5-h induction at 30 °C, cultures were centrifuged and resuspended in 1× SDS sample buffer and boiled for 10 min. Lysates were cleared by centrifugation at 10,000 rpm for 5 min, and aliquots of lysate were subjected to either SDS-PAGE and Western blotting using antibodies specific for FUS, CHOP, EWS, and FLI1 or subjected to Elisa Microplate Assay using antibodies specific for FUS, CHOP, EWS, and FLI1. Blots were processed using a LI-COR Biosciences Odyssey Fc imaging system.

For FRAP experiments, yeast were harvested after 5-h induction at 30 °C. Yeast were immobilized on slides using a 4% agarose pad supplemented with media and sealed with nail polish. Images were acquired using a Zeiss LSM 880 Airyscan confocal microscope, which was purchased with support from the Office of Research Infrastructure Programs (ORIP), a part of the National Institutes of Health Office of the Director, under grant OD021629.

Acknowledgments—We thank Peter Bayguinov and James Fitzpatrick of the Washington University Center for Cellular Imaging for assistance with microscopy. We thank Zach March, Korrie Mack, and Bede Portz for review of the manuscript and Jeffrey Toretsky for providing plasmids. Confocal data were generated on a Zeiss LSM 880 Airyscan confocal microscope, which was purchased with support from the Office of Research Infrastructure Programs (ORIP), a part of the National Institutes of Health Office of the Director, under grant OD021629.

References


Enhanced protein disaggregases to counter sarcoma


