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as of 13-Oct-2022

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Final Report for Period Beginning 01-Dec-2018 and Ending 30-Nov-2021
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Authors: Michael Veling, Dan Nguyen, Nicole Thadani, Michela Oster, Nathan Rollins, Kelly Brock, Neville Bethel
Keywords: Stress tolerance, Phase separation, Intrinsically disordered proteins, Apoptosis
Abstract: Many organisms can survive extreme conditions and successfully recover to normal life. This extremotolerant behavior has been attributed in part to repetitive, amphipathic, and intrinsically disordered proteins that are upregulated in the protected state. Here, we assemble a library of approximately 300 naturally-occurring and designed extremotolerance-associated proteins to assess their ability to protect human cells from chemically-induced apoptosis. We show that proteins from tardigrades, nematodes, and the Chinese giant salamander are apoptosis protective. Notably, we identify a region of the human ApoE protein with similarity to extremotolerance-associated proteins that also protects against apoptosis. This region mirrors the phase separation behavior seen with such proteins, like the tardigrade protein CAHS2. Moreover, we identify a synthetic protein, DHR81, that shares this combination of elevated phase separation propensity and apoptosis protection. Finally, we demonstrate that
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Article Title: Natural and designed proteins inspired by extremotolerant organisms can form condensates and attenuate apoptosis in human cells
Authors: Mike Veling, Dan Nguyen, Nicole Thadani, Michela Oster, Nathan Rollins, Kelly Brock, Neville Bethel, D
Keywords: IDPs, apoptosis
Abstract: Many organisms can survive extreme conditions and successfully recover to normal life. This extremotolerant behavior has been attributed in part to repetitive, amphipathic, and intrinsically disordered proteins that are upregulated in the protected state. Here, we assemble a library of approximately 300 naturally-occurring and designed extremotolerance-associated proteins to assess their ability to protect human cells from chemically-induced apoptosis. We show that proteins from tardigrades, nematodes, and the Chinese giant salamander are apoptosis protective. Notably, we identify a region of the human ApoE protein with similarity to extremotolerance-associated proteins that also protects against apoptosis. This region mirrors the phase separation behavior seen with such proteins, like the tardigrade protein CAHS2. Moreover, we identify a synthetic protein, DHR81, that shares this combination of elevated phase separation propensity and apoptosis protection. Finally, we demonstrate that
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Article Title: Multi-omics analysis of Dsup expressing human cells reveals open chromatin architectural dynamics underyling radioprotection
Authors: Craig Westover, Deena Najjar, Cem Meydan, Kirill Grigorev, Mark T Veling, Roger L Chang, Christophe
Keywords: Dsup, tardigrade, chromatin, radioprotection, reactive oxygen species
Abstract: Spaceflight has been documented to produce detrimental effects to physiology and genomic stability, partly a result of Galactic Cosmic Radiation (GCR). In recent years, extensive research into extremotolerant organisms has begun to reveal how they survive harsh conditions, such as ionizing radiation. One such organism is the tardigrade (Ramazzottius varieornatus) which can survive up to 5kGy of ionizing radiation and the vacuum of space. In addition to their extensive network of DNA damage response mechanisms, the tardigrade also possesses a unique damage suppressor protein (Dsup) that co-localizes with chromatin in both tardigrade and transduced human cells to protect against DNA damage from reactive oxygen species induced by ionizing radiation. While Dsup has been shown to confer human cells with increased radiotolerance; much of the mechanism of how it does this in the context of human cells remains unknown. Until now there is no knowledge yet of how introduction of Dsup into human
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RPPR Final Report
as of 13-Oct-2022

Partners

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I certify that the information in the report is complete and accurate:
Signature: Roger Chang
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Harvard Medical School/
DARPA Biostasis
Final Technical/ R&D Status Report

Period Covered by the Report: 12/1/2018 through 11/30/2021

Date of Report: 2/28/2022
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Contract or Agreement Number: W911NF-19-2-0017
Total Dollar Value: $6,513,722
Subcontractors: Massachusetts Institute of Technology, University of Washington
Program Manager: Dr. Tristan McClure-Begley, DARPA/BTO

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1 High-Level Project Progress, including Big Wins

Intrinsically disordered proteins with protective properties exhibit amphipathic helical repeats

In our initial library of ~100 IDPs derived from extremotolerant organisms, we observed through sequence analysis that most of these proteins exhibit a repeated helix-linker-helix motif, in which the helical regions have notable amphipathic character with polar residues enriched on one side and non-polar residues enriched on the opposing side. We hypothesized that this motif indicated a sticker-spacer structure permitting self-interaction that could lead to formation of higher-order structures from these IDPs. This motivated our design of synthetic variants isolating these repeat regions, mutating the helices to test specific hypotheses regarding potential interactions, identifying proteins with similar motifs in the human proteome, and developing completely synthetic sequences with the same motif that can self-interact. Throughout the course of this project, we assembled a gene expression construct library of ~500 of these naturally-occurring and designed sequences to be tested in functional assays as candidate biostasis proteins.

Diverse IDPs protect human cells from stress, slow cellular processes, and promote conditions consistent with dormancy in hibernating species

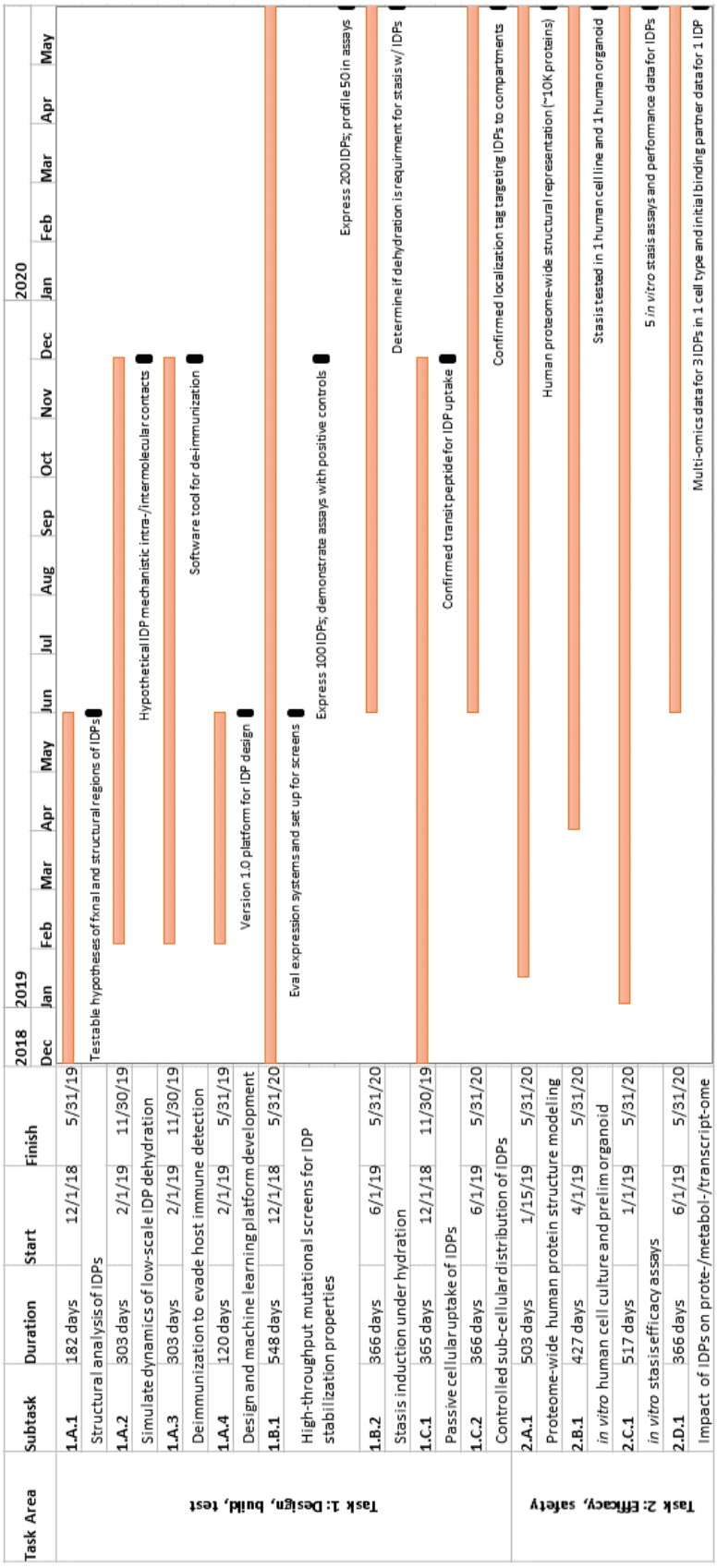
We developed 12 efficacy assays by which to screen our library of candidate biostasis IDPs. Assays showing significant positive results for stabilizing properties of candidates included most notably: apoptosis, hypoxia response, innate immune response, and enzyme stabilization. A high percentage of our IDP library showed anti-apoptotic effects via a reduction in Caspase activity; the diverse best performers included variants of Apolipoprotein E (APOE), a synthetic designed helical repeat protein (DHR81), multiple late embryogenesis abundant (LEA) proteins, and tardigrade-specific IDPs CAHS and DSUP. LEA proteins, especially those derived from the polyextremophile bacterium *Deinococcus radiodurans*, led to increased hypoxia response, which is notable given that hypoxia is a common feature of animals in torpor or hibernation states. We also found that several candidates slowed innate immune response as measured by STAT1 nuclear translocation and many candidates stabilized cell-free lactate dehydrogenase (LDH) enzyme against thermal stress.

Most-protective IDPs form phase-separated condensates as part of their mechanism of action

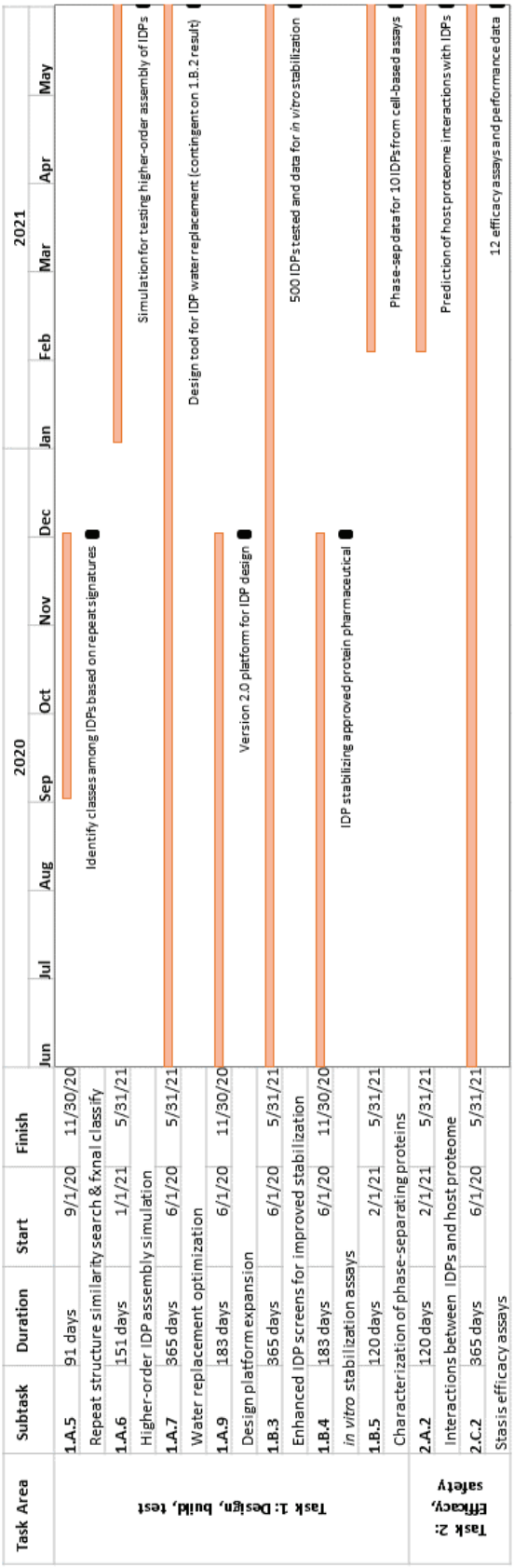
Following-up on the original hypothesis that the formation of higher-order structures by IDPs is related to their stabilizing properties and our observation that many candidate IDPs form gels in relatively pure and hydrated samples, we observed by microscopy whether our best-performing candidates in the apoptosis assay readily formed protein condensates in human cells. Indeed, several did exhibit protein condensate formation; thus, we developed a synthetic biology strategy to test whether the anti-apoptotic property of these IDPs was a direct consequence of their ability to form condensates. Fusing these IDPs to an inducible-multimerizing domain, we determined that we could kinetically promote condensate formation in a controlled manner. Using this system, we found that condensate formation of APOE, DHR81, and LEA proteins concomitantly led to a decrease in apoptotic pathway activity, confirming that the propensity of these proteins to form higher-order structures takes part directly in their stabilizing mechanism. Conversely, although CAHS readily forms condensates and can be induced to do so in our synthetic system, this induction did not lead to concomitant decrease in Caspase activity. So although diverse IDPs do function at least in part by forming higher order structures, others must have distinct mechanisms of protection.

2 Schedule: Milestones and Deliverables

Phase 1 Gantt Chart



Phase 2 Gantt Chart



3 Tasks: Progress, Accomplishments, and Plans

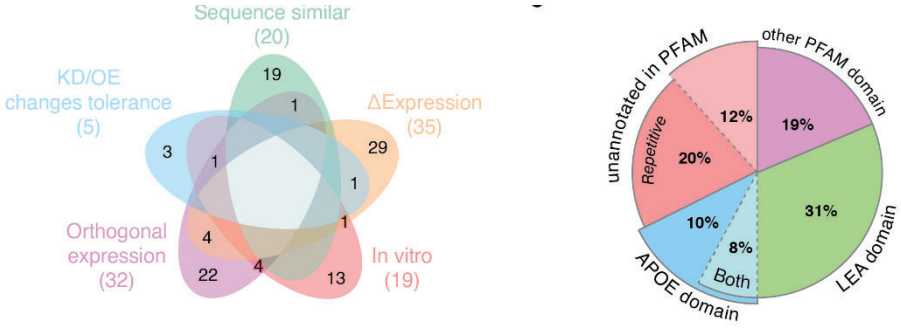
Task #/Title	Task Description	% Complete	Total Spent	Explain deviations between Planned vs. Actual
P1: 1.A.1	Testable hypotheses of functional and structural sub-regions of IDPs	100%	$584,440.91	
P1: 1.A.2	Hypothetical IDP mechanistic intra-/intermolecular contacts	100%	$373,807.17	
P1: 1.A.3	Software tool for de-immunization to tune for safety	100%	$105,316.87	
P1: 1.A.4	First version of software platform for IDP design for iterative improvement	100%	$210,633.74	
P1: 1.B.1	Evaluate expression systems and set up for screening of vitrification properties	100%	$368,660.69	
P1: 1.B.2	Determine feasibility of stasis induction under hydration	100%	$414,479.63	
P1: 1.C.1	≥1 confirmed transit peptide for protein uptake and rough bounds for size	100%	$109,387.12	
P1: 1.C.2	≥1 confirmed sub-cellular localization tag	100%	$98,937.36	
P1: 2.A.1	Human proteome-wide structural representation (>10,000 protein species)	100%	$215,941.50	
P1: 2.B.1	Stasis tested in ≥1 human cell line and 1 human organoid	100%	$587,500.79	
P1: 2.C.1	5 in vitro stasis assays and performance data for IDPs	100%	$785,938.06	
P1: 2.D.1	Proteomic/metabolomic/transcriptomic data for ≥3 IDPs in ≥1 cell type and 1 organoid; binding partner data for ≥1 IDP in ≥1 cell type	100%	$162,183.15	
P2: 1.A.5	Repeat structure	100%	$146,338.13	
P2: 1.A.6	Simulate intermediate-scale CryP assemblies	50%	$126,556.33	
P2: 1.A.7	Water replacement optimization	100%*	$167,606.17	*property deemed unnecessary
P2: 1.A.9	Expanded design platform for iterative improvement	100%	$200,255.89	
P2: 1.B.3	Enhanced CryP screens for improved stabilization	91%	$419,192.40	
P2: 1.B.4	In vitro stabilization of approved protein pharmaceutical	35%	$198,123.59	
P2: 1.B.5	Phase-sep	100%	$182,133.77	
P2: 2.A.2	Predict interactions between IDPs and host proteome	25%	$172,763.73	
P2: 2.C.2	12 stasis assays and CryP performance data	80%	$883,523.99	

Assembling and characterizing a library of candidate biostasis IDPs

We assembled an initial list of ~100 IDPs associated with extremotolerance derived from extremophiles such as tardigrades and other species that can undergo cryptobiosis. The criteria

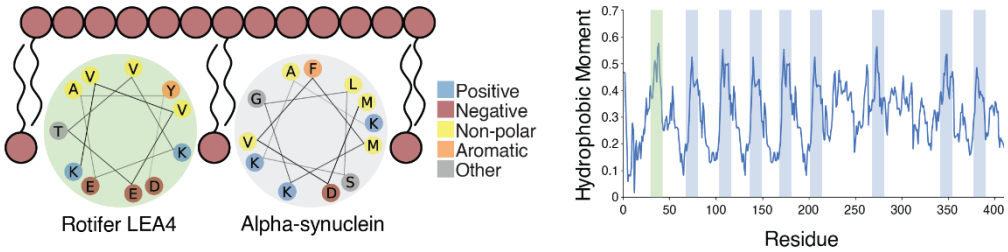
for inclusion in this list (Figure 1 left) included evidence for heterologous expression transferring a protective phenotype to another species, cell-free *in vitro* protection of function of a purified enzyme, gene knockout or overexpression experiments leading to stress resistance phenotypes, changes in endogenous expression concomitant with onset of environmental stress, and human proteins with sequence similarity to IDPs selected due to previous criteria. In our initial attempt to characterize common features among these proteins (Figure 1 right), we assigned protein families (PFAMs) to all of these sequences and found that primarily late embryogenesis abundant (LEA) proteins and apolipoprotein E (APOE) domains were present in about half of them.

Figure 1



Seeing that PFAM annotation was insufficient to unify this list of proteins, we looked for more specific sequence motifs. As others have previously observed the presence of a repeat motif in some of these IDPs, we too searched comprehensively for repeats within our initial list and uncovered a common general motif of helix-linker-helix (example in Figure 2 right), where the helices are amphipathic in that they are enriched in polar residues on one side and non-polar residues on the opposite side. Such amphipathic helices are known to interact with other surfaces in the cell, such as membranes or other proteins (examples in Figure 2 left). Furthermore, the charged residues on these helices suggested the possible inter-/intramolecular parallel or anti-parallel helix-helix interactions that might lead to formation of higher-order structures from these proteins.

Figure 2



Because these helix-linker-helix repeats across proteins in our initial list were not necessarily homologous, we developed our own spectrogram analysis approach to look for more degenerate patterns that might unify or at least help us to classify these proteins in a useful manner **(Task 1.A.5)**. Although we were able to use this new approach to relate the repeats

across these proteins, we were ultimately unable to make sense of the observed patterns in terms of eventual assay performance of these proteins.

From this initial candidate list of IDP sequences, we assembled a gene expression construct library (one for human cell expression and another for *E. coli* **(Tasks 1.B.1 and 1.B.3)**), including not only the naturally-occurring IDPs but also truncations and other mutations thereof and completely synthetic repeat proteins designed to self-interact. For proteins with a sufficient number of homologous sequences, we developed statistical conservation models (EV models) from which to select some of the mutants we would test for stasis properties **(Task 1.A.1)**. In addition, in anticipation of eventual animal studies and development of a therapeutic for human use, we developed a software tool for decreasing the immunogenicity of synthetic IDPs, removing potential epitope sequences while attempting to preserve functionality of the original sequences **(Task 1.A.3)**. Upon obtaining performance data in stabilization assays (description to follow), we iteratively returned to this design step to look for guiding principles that might inform these subsequent designs. In general, the main principle we found was that the repeat regions were essential for any stabilizing effects and that the more extensive the repeat regions and less non-repeat sequence included from the original proteins, the stronger any stabilizing effects **(Tasks 1.A.4 and 1.A.9)**.

Milestones by task
1.A.1 Structural analysis of IDPs
Milestone: Testable hypotheses of functional and structural sub-regions.
Progress: Milestone achieved.

1.A.3 De-immunization to evade host immune detection
Milestone: Software tool for de-immunization to tune for safety.
Progress: Milestone achieved.

1.A.4 Design and machine learning platform development
Milestone: First version of software platform for IDP design for iterative improvement.
Progress: Milestone achieved.

1.B.1 High-throughput mutational screens for IDP stabilization properties
Milestone: At least 200 IDP variants and mutants synthesized in microbial or mammalian expression systems and performance data for structural protein features, including expression levels and bounds on minimal stasis domains to use in design, and basic biochemical profiling (such as thermal solubility, NanoDSF, and MW analysis). This will be done in iterative rounds of design and screening to permit sampling a breadth of starting proteins and then beginning to drill-down into variants and similar proteins to those showing promise in the previous iteration. (Month 6: evaluate expression systems and set up for screening of stabilization properties. Month 12: express 100 wild-type and variant proteins; demonstrate our assay works with positive controls. Month 17: Express at least 200 such proteins. Month 18: Profile at least 50 such proteins in all assays.)
Progress: Milestone achieved.

1.A.5 Repeat structure of IDPs for similarity searches and functional classification
Milestone: Identified functional classes among natural and synthetic IDPs on the basis of their repeat signatures.
Progress: Milestone achieved, but there essentially were no discernable distinct functional classes that could be found by differing repeat signatures. There was really just one class of stabilizing proteins that share a generic repeat structure.

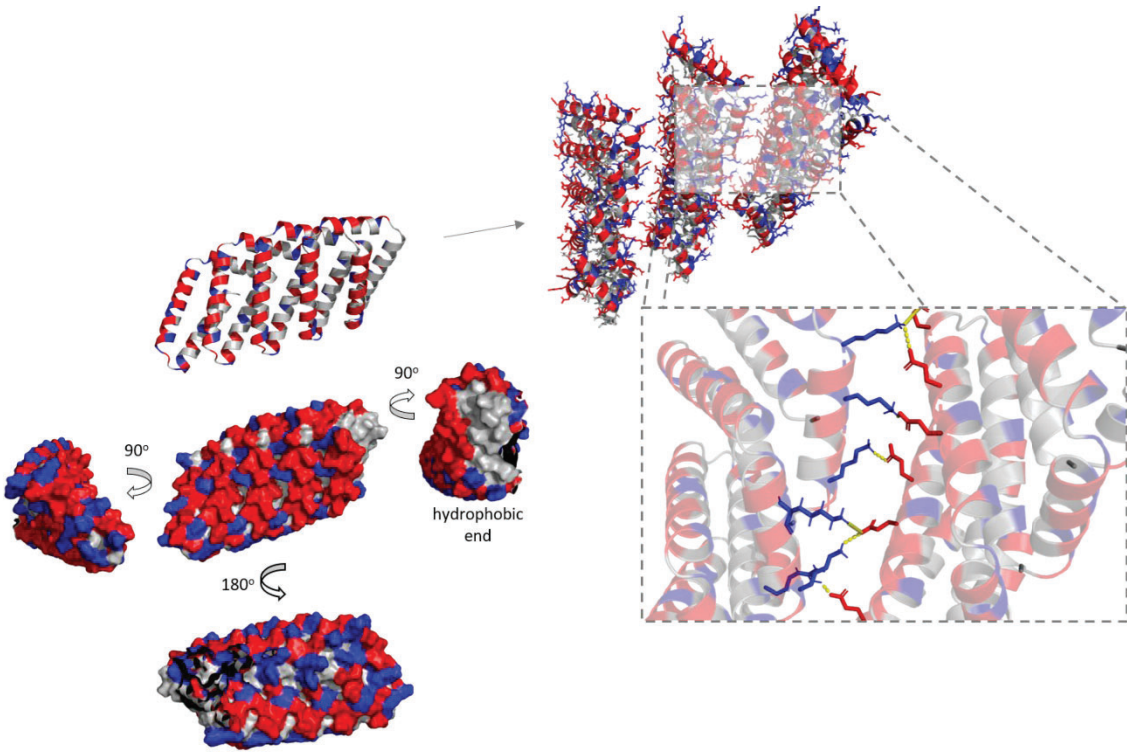
1.A.9 Expansion of design and machine learning platform
Milestone: Second version of software platform for IDP design for iterative improvement.
Progress: Milestone achieved.

1.B.3 High-throughput enhanced IDP design screens for improved stabilization
Milestone: At least 500 new IDPs synthesized and in vitro stabilization data.
Progress: Milestone achieved.

Theoretical models of IDP interactions

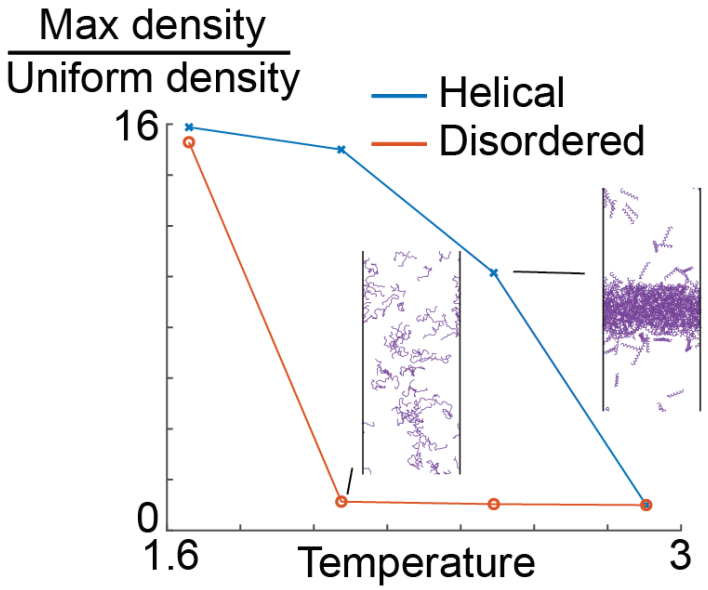
Since from literature we already knew that some of the naturally-occurring IDPs with protective effects form higher-order structures that present as gels (e.g. tardigrade CAHS proteins), we sought to model and perhaps uncover the molecular interactions that support this phenomenon. We first developed static 3D models of multimerization of a longer (DHR81) and a shorter (PvLEA-22) synthetic repeat IDP **(Task 1.A.2)**. For example (Figure 3), we modeled a monomer of the designed helical repeat protein DHR81 in which the intramolecular helix-helix interactions are clear across the repeats. This protein exhibited hydrophobic ends that might facilitate intermolecular interactions across multiple copies of the protein but also exhibit complementary charge distributions along the lateral surfaces, which could also be expected to permit a mode for intermolecular interactions across multiple copies. The expectation is that these interactions permit formation of an extended network of interacting copies of DHR81 that can be observed as aggregates or perhaps a gel in experiments (to be presented later).

Figure 3



We also sought to simulate the dynamics of even higher order (~500 molecules) interactions among a population of the same species of IDP through coarse-grained modeling. We developed such a model for the model human IDP FUS as well as one of the shorter sequences from our library of stasis candidates (PvLEA-22) **(Task 1.A.6)**. For PvLEA-22, our simulations suggested that the completely disordered state of the protein did not as readily facilitate condensation, and therefore higher-order structure formation, as when the helical secondary structure was induced (Figure 4). This is because the helix formation was required to create amphipathic faces with the potential for self-interaction. Therefore, we hypothesized that despite these IDPs being largely disordered as isolated monomers, they could be expected to have formed interacting helices when observed in a material state in subsequent experiments (to be presented below).

Figure 4



Beyond self-interactions, we were interested in the possibility that candidate stasis IDPs might interact with other proteins in host cells, perhaps as part of their assembly into higher-order structures and/or perhaps to target specific or non-specific orthogonal proteins for stabilization as part of their protection mechanisms against stress. To this end and because we would be testing these IDPs in human cells, we sought first to structurally model as much of the human proteome as possible. To begin with, we developed a new EV server and generated EV models for ~12,000 human proteins **(Task 2.A.1)**. Furthermore, we also employed a homology-modeling approach to model ~7,000 proteins that have been detected in one or both of the two main human cell types we implemented in stabilization assays (HT-1080 and HEK293 cells) **(Task 2.A.1)**. We also catalogued the known interactions between human intrinsically disordered regions (IDRs) and globular domains based on literature and protein-protein interaction databases as a starting point for predicting how other IDPs might interact with the human proteome **(Task 2.A.2)**. Unfortunately, we did not get sufficient data to develop these predictions further. However, we have uploaded the models we generated for human proteins to a publicly accessible server (https://doi.org/10.5061/dryad.1jwstqjxw).

Milestones by task

1.A.2 Simulate dynamics of low-scale IDP dehydration
Milestone: Hypothetical IDP mechanistic intra-/intermolecular contacts.
Progress: Molecular contacts generated for a longer and a shorter repeat protein based on low-scale interactions of a static model.

2.A.1 Proteome-wide human protein structure modeling
Milestone: Human proteome-wide structural representation, including >10,000 distinct protein species.
Progress: Milestone achieved.

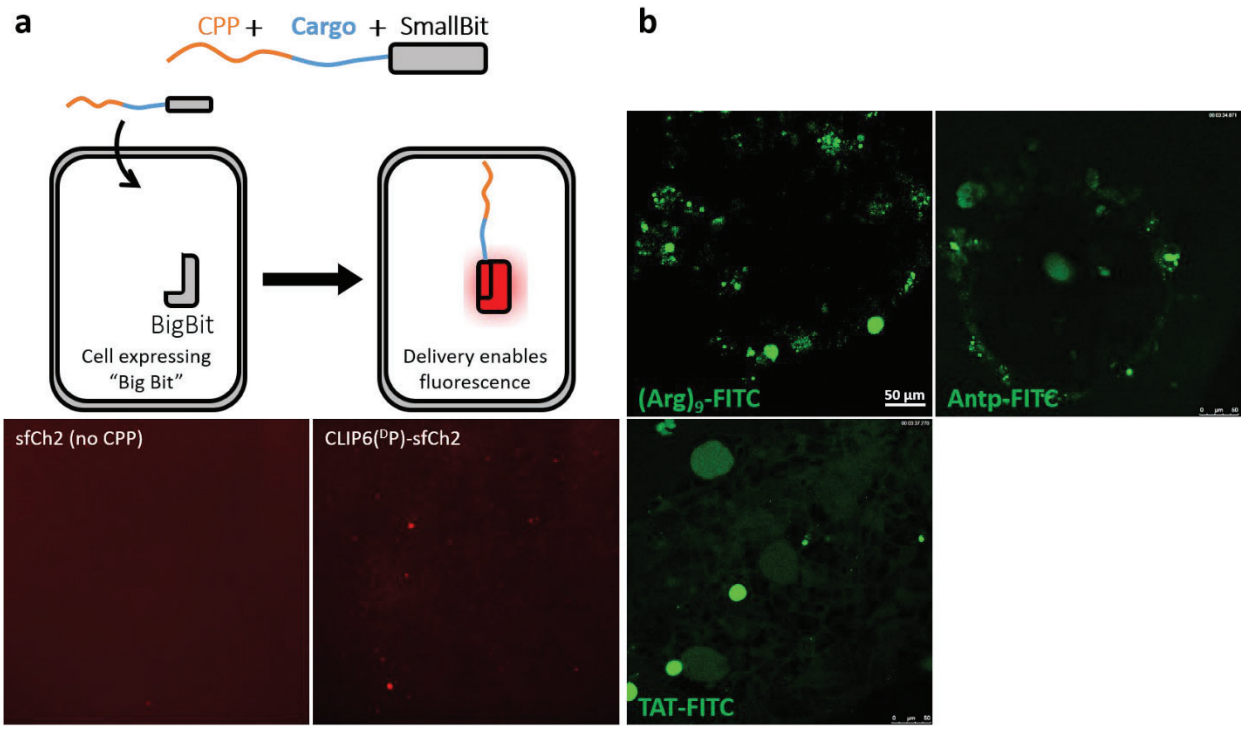
1.A.6 Develop higher-order assembly simulation capability and then simulate hydrated intermediate-scale IDP assemblies
Milestone: Software tool for testing matrix assembly of IDP designs.
Progress: A tool per se was did not result; however, intermediate-scale dynamic simulations were performed for 2 IDPs (human FUS and PvLEA-22) using coarse-grained modeling.

2.A.2 Develop framework to predict interactions between IDPs and the host proteome
Milestone: Software tool for prediction of host proteome interaction with IDPs and framework by which to integrate and enrich interaction proteomics data. The tool will be used predict interactions between the human proteome and all designed biostasis IDP candidates.
Progress: A tool was not developed. All available published data were collected concerning IDP/IDR interactions with human proteins for this purpose, but there was insufficient data to develop a generalizable predictive model.

Characterize and develop delivery, localization, and stability properties of biostasis IDPs

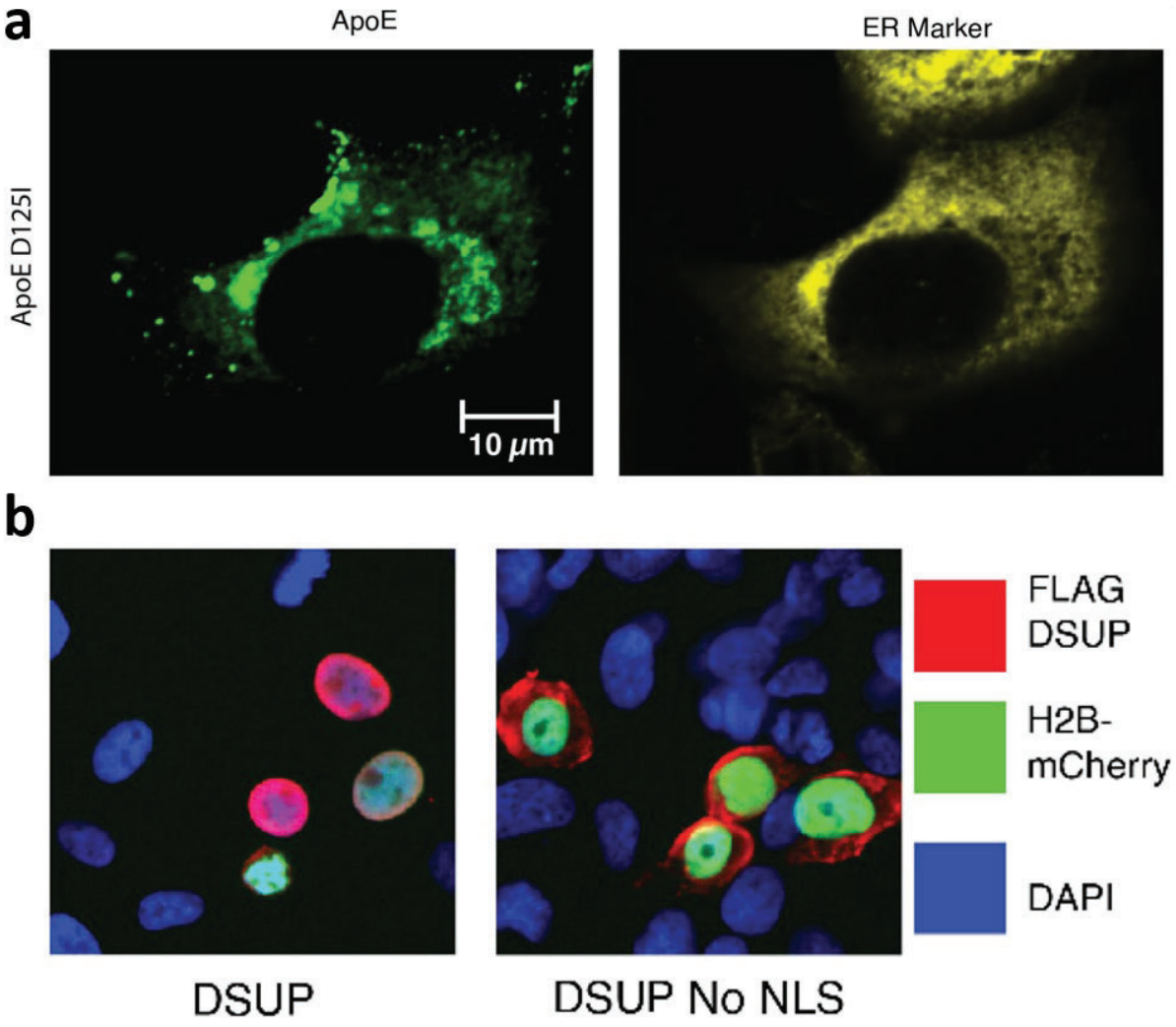
To prepare for delivery of our eventual biostasis IDP candidate(s) to tissues *in vivo*, we tested a cell-penetrating peptide (CPP) strategy to delivery proteins into human cells. CPPs are generally positive-charge-enriched N-terminal peptides that facilitate uptake into cells by multiple mechanisms. We used a split-mCherry construct to test for cellular uptake of CPP-fused cargo into HEK293 cells expressing the larger portion (BigBit) of mCherry and fusing the smaller portion (SmallBit) to the CPP. The CLIP6(^{D}P) worked to deliver a detectable quantity of SmallBit into these cells (Figure 5a), and we saw similar results for the CPP TAT as well. We also tested how CPPs would work in a multicellular tissue setting by introducing CPP-FITC conjugates to liver organoids through growth medium. To varying extents, $(Arg)_9$, Antp, and TAT all successfully delivered cargo into at least the outer layer of cells in the organoids (Figure 5b). Therefore, we concluded that CPPs indeed function as a vehicle for delivery of generic cargo **(Task 1.C.1)**, though we did not proceed to use them to introduce IDPs to cells in our high-throughput stabilization assays due to time and resource efficiency.

Figure 5



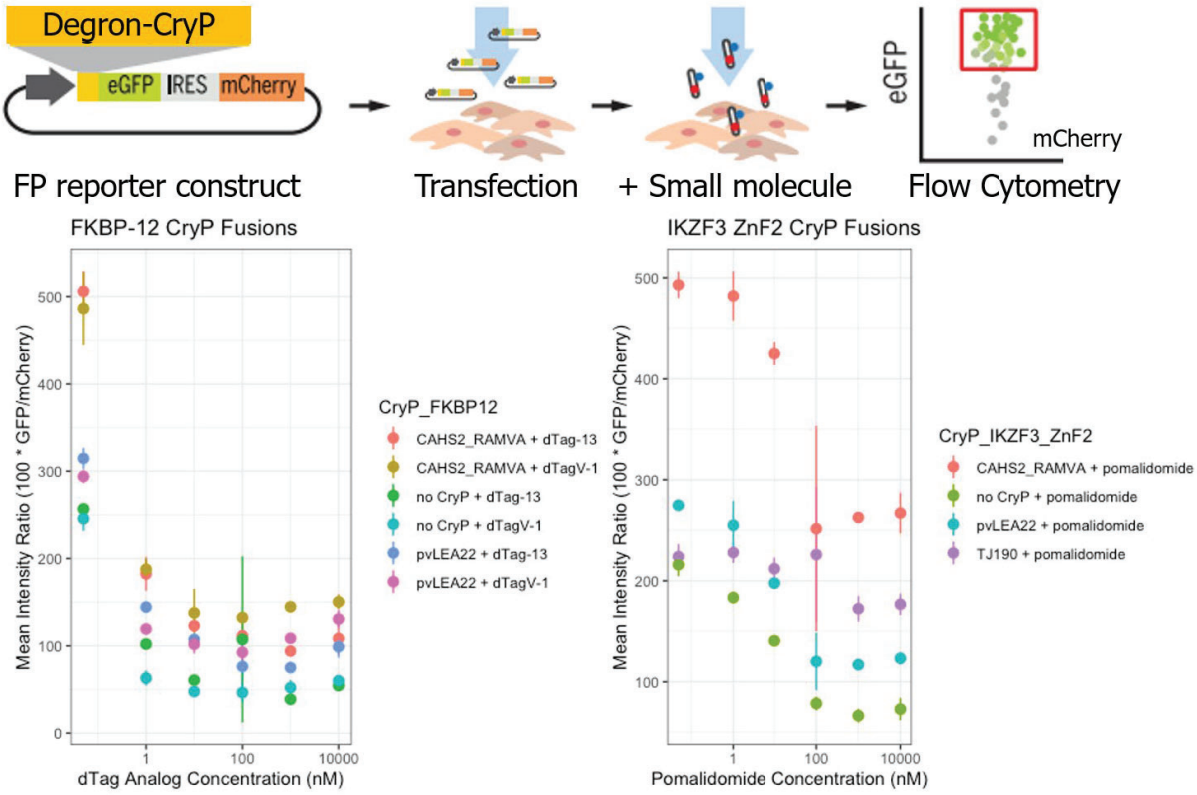
Given our initial lack of knowledge about where IDPs might need to distribute within cells in order to elicit a stabilizing phenotype, we initially thought that we might need to be able to target our IDPs any or all of several subcellular compartments. As will be subsequently shown, stabilization phenotypes are achievable from at least many IDPs in our library simply localizing broadly to the cytosol. However, we did characterize and experiment with modulating the localization of some of our candidates based on the presence or truncation of endogenous localization signals **(Task 1.C.2)**. For example, human APOE protein is typically a secreted protein and therefore often found in the extracellular space; however, we designed truncations of APOE removing the secretion signal, which resulted in the rest of the APOE protein remaining in the cell and roughly localizing to the ER (Figure 6a). We also demonstrated the nuclear localization of tardigrade damage suppressor protein (DSUP) in its full form but cytosolic localization in truncations removing the nuclear localization signal (NLS) (Figure 6b). We also tested the effect of replacing the endogenous NLS of DSUP with ones derived from human proteins, but to our surprise, these variants did not reach the nucleus and remained in the cytoplasm (data not shown).

Figure 6



To facilitate reversal of stasis conditions conferred by IDPs, we strategized to use controlled degradation by way of either modulating the half-life of IDPs (e.g. N-end rule) or inducible degradation using degrons fused to our candidates **(Task 2.A.4)**. We developed a system to measure degradation rates of a degron-IDP-FP fusion expressed in a single transcript with and relative to a second fluorescent protein not fused to any degron (Figure 7 top). Three different chemically inducible degrons were tested in this system with three different cargo IDPs, and fluorescent signals indicating protein concentration in expressing cells were measured by flow cytometry at different concentrations of degron inducers. Clear degradation curves were observed (Figure 7 bottom), demonstrating that the inducible degradation of cargo IDPs had been achieved. Although we did not implement these systems in tandem with our stabilization assays, this strategy is very likely to be successful in such applications based on our observations.

Figure 7



Milestones by task

1.C.1 Passive cellular uptake of IDPs
Milestone: ≥1 confirmed transit peptide to achieve passive uptake of synthetic IDP designs and rough bounds for size constraints for fusion uptake.
Progress: Milestone achieved.

1.C.2 Controlled sub-cellular distribution of IDPs
Milestone: ≥1 confirmed localization tag targeting IDPs to compartments including the cytosol, cell membrane, nucleus, mitochondria, endoplasmic reticulum, or Golgi.
Progress: We confirmed localization tags for nucleus and secretion and tagless proteins localizing to cytosol; however, it was eventually apparent that cytosolic localization was sufficient to observe stabilized phenotypes in functional assays.

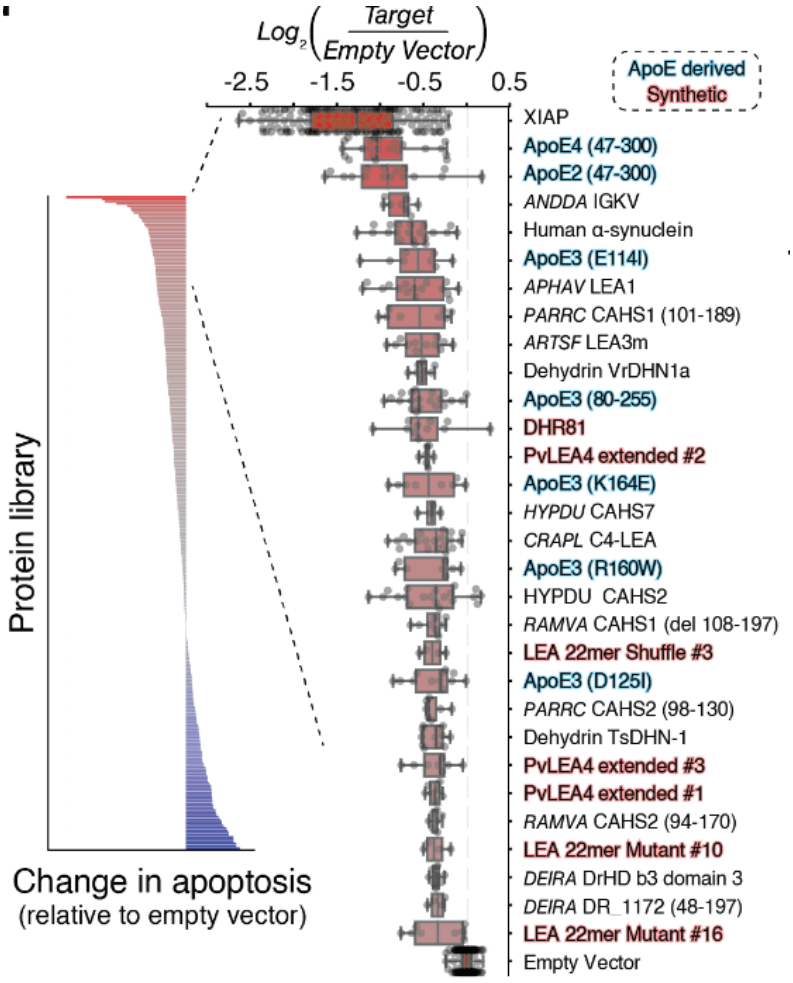
**2.A.4 Controlled degradation of IDPs for stasis reversal*
Milestone: ≥1 confirmed degradation tag with enhanced stasis reversal kinetics of synthetic IDP designs.
Progress: Developed degron-based system for controlled IDP degradation, but this sub-task was eliminated from the SOW by the program before we could proceed further characterizing this.

Establishing efficacy of candidate biostasis IDPs in stabilizing biological systems

The most important portion of our proposal was to establish whether any of our ~500 candidate biostasis IDPs actually have slowing and/or stabilizing effects on biological systems, especially human cells. Therefore, we developed 12 stabilization assays to evaluate this efficacy for our protein library: enzyme stabilization (LDH), immune response (STAT1, NFkB), apoptosis, RBC stabilization, phase-separation, hypoxia response, cytoskeleton stability, cell drying, hepatocyte urea synthesis, organoid hyperosmotic stress tolerance, and tissue plasminogen activator stabilization **(Tasks 2.C.1 and 2.C.2)**. Some of these were performed cell-free, some in simple 2D cell culture, and some in organoids **(Task 2.B.1)**. Positive results from some of these assays are highlighted below. We report the full quantitative results from these assays, including fold changes for effect size, in the supplementary file “Biostasis assay results 8-11-2022.xlsx” that was provided by email to the DARPA team.

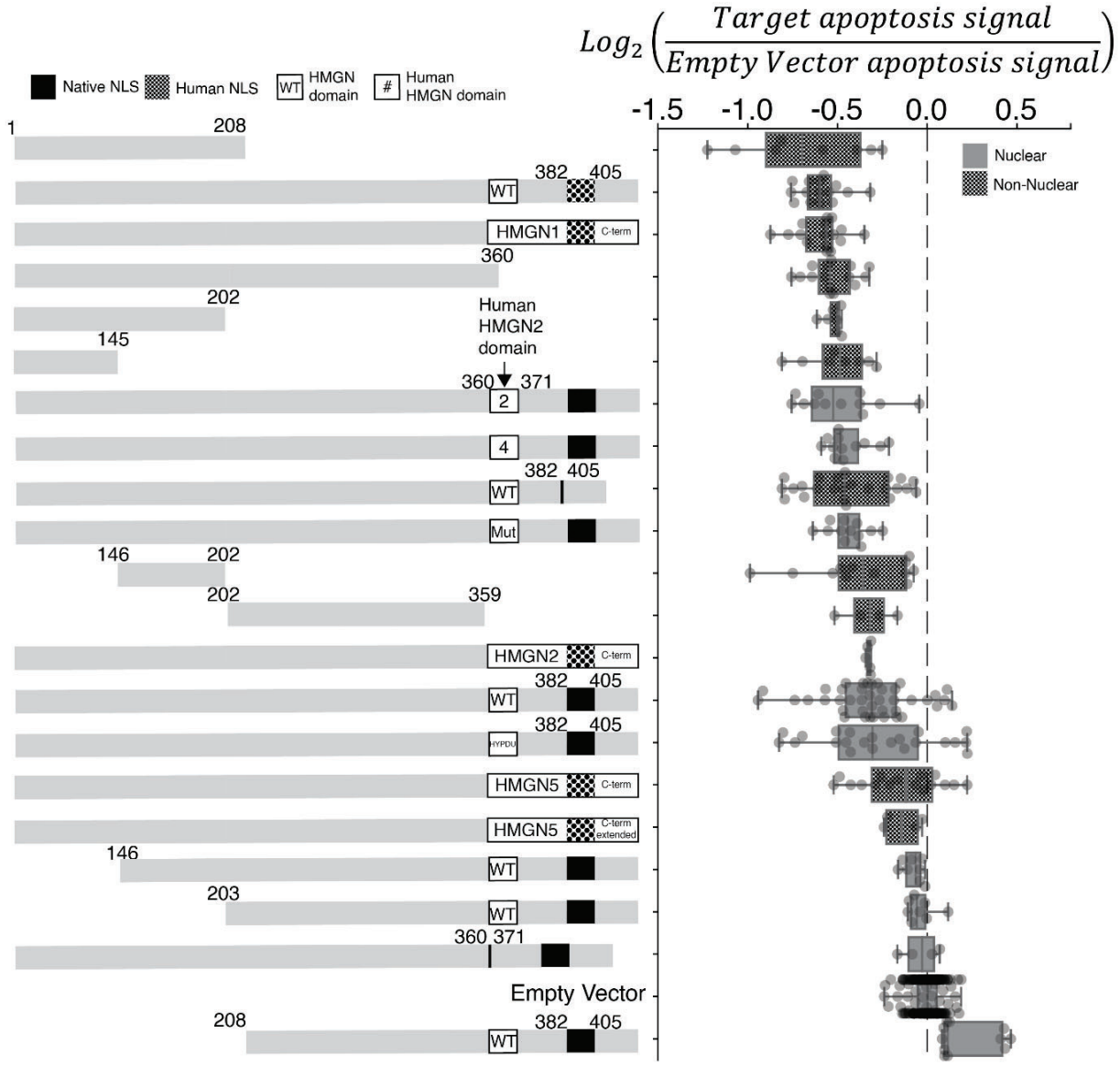
The assay that we ultimately observed the most reproducible positive results for was the apoptosis assay, in which Caspase 3/7 activity was measured in living cells by microscopy time course over a 24-hour period and the extent of apoptotic activity was compared to an empty vector control to establish degree of anti-apoptotic efficacy. Across our library, the majority of IDPs we tested showed at least some degree of anti-apoptotic activity (top performers are highlighted in Figure 8). Some diverse proteins are represented among the top performing IDPs in this assay, including variants of the repeat region from human APOE, several LEAs, some CAHSs, the synthetic DHR81, and DSUP variants.

Figure 8



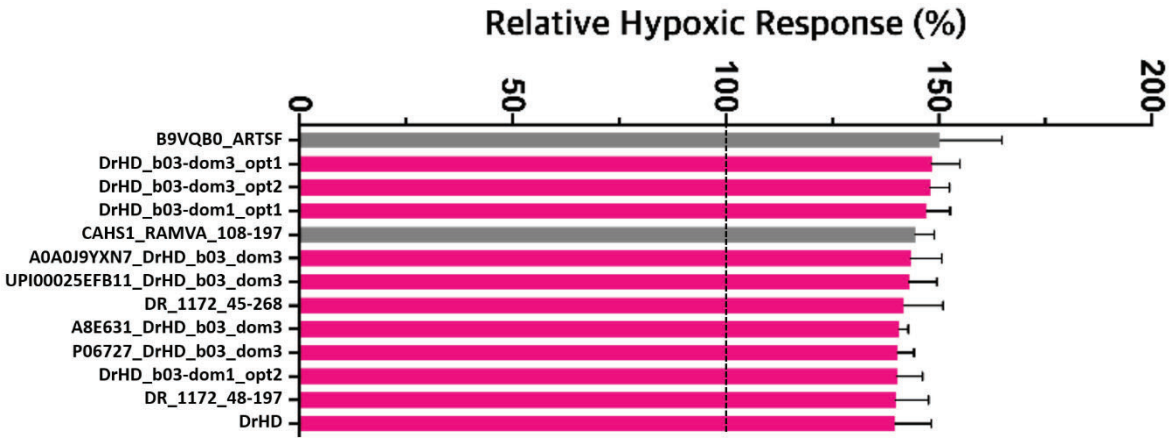
Following-up on the observation that both WT DSUP and truncations missing the NLS were efficacious in the apoptosis assay, we designed many synthetic DSUP variants modulating the length of the disordered region included and exchanging the NLS and nucleosome binding domain for homologous sequences from other tardigrade species and human proteins. Surprisingly, we observed that the most effective DSUP variants lacked the native NLS and therefore were not transported to the nucleus (Figure 9). Therefore, we have discovered a perhaps latent cytosolic stabilizing function of the DSUP protein.

Figure 9



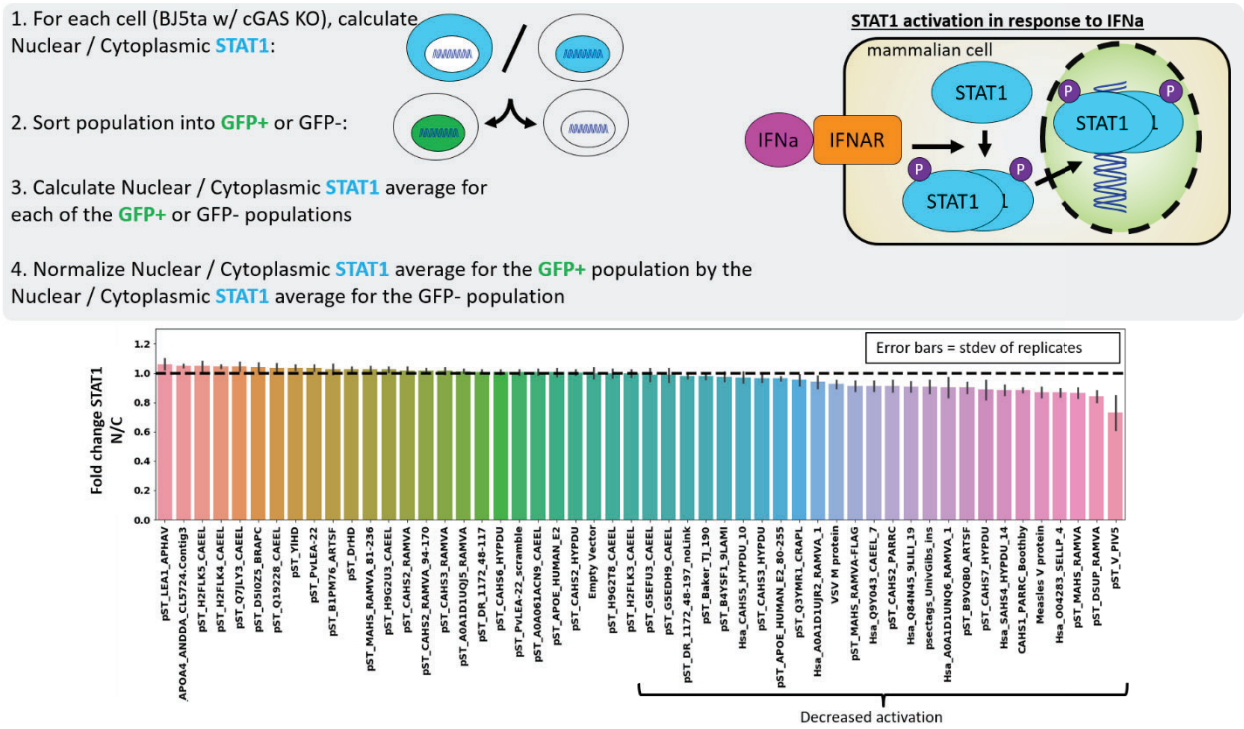
In another efficacy assay, we used a transcriptional reporter indicating activity of the hif1a-mediated pathway for hypoxic response. Hypoxia is a hallmark of cellular states in animals during torpor and hibernation. Most of our IDP library did not affect hypoxia response; however, practically every synthetic variant of the LEA protein DR_1172 derived from the polyextremophile bacterium *Deinococcus radiodurans* resulted in an increased hypoxia response in human cells (Figure 10).

Figure 10



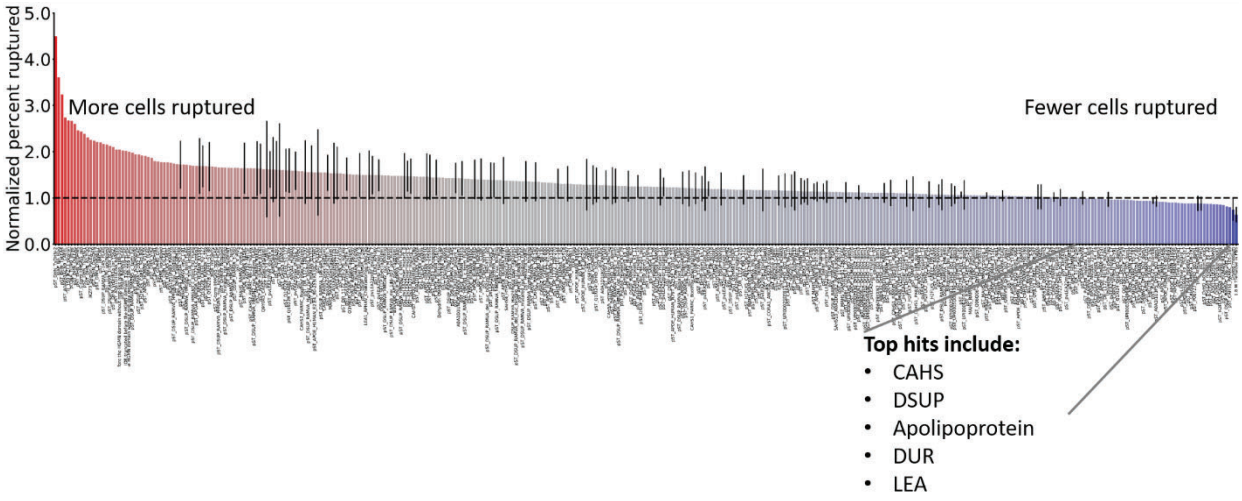
To evaluate whether our candidate IDPs might slow innate immune response, we developed two assays, one for STAT1 nuclear translocation and one for NFkB activation, both of which result from cellular exposure to foreign materials. The STAT1 assay measures STAT1 nuclear translocation by microscopy following treatment with IFNa, and expected stabilization effects were observed as decreased translocation with expression of candidate IDPs (Figure 11). We observed that primarily tardigrade-specific IDPs (e.g. DSUP, MAHS, and CAHS) had a slowing effect on activity of this pathway.

Figure 11



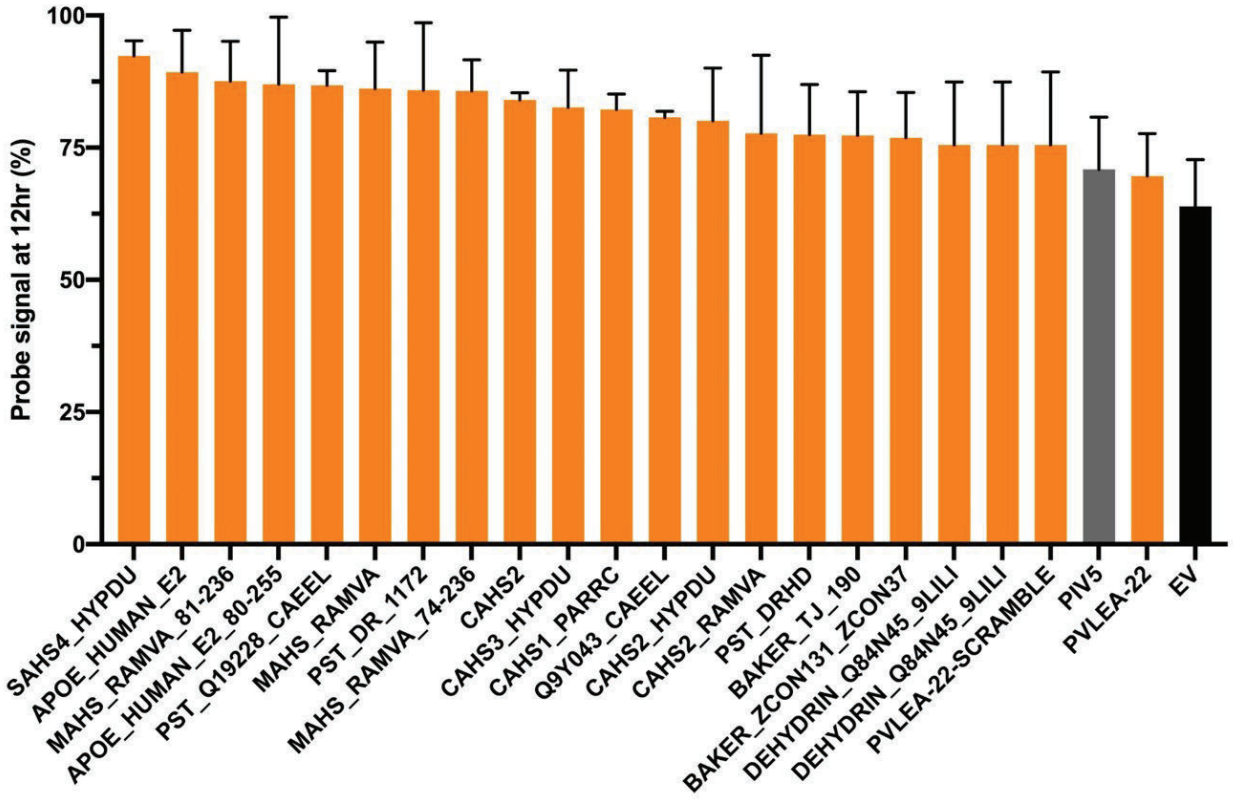
We tested our library of IDPs in protecting human cells from rupturing due to drying as well (Figure 12). Most of our candidates actually increased the rate of cell rupture during drying, but a small number of them showed a slight protective effect (e.g. CAHS, DSUP, APOE, LEA, and a dauer-upregulated protein DUR from *C. elegans*). As can be observed from the error bars in this data, reproducibility was often very challenging, and so we relied less on this assay in distinguishing protective IDPs.

Figure 12



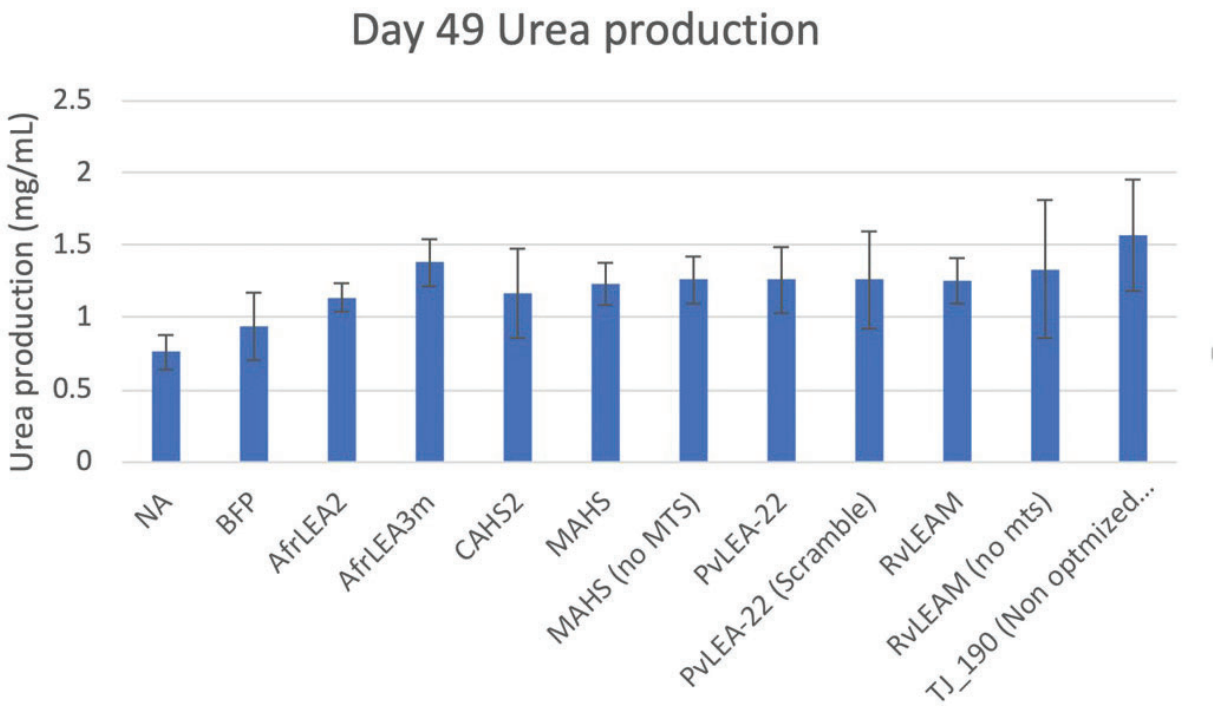
The ability of our candidates to stabilize cellular structure was evaluated in a cytoskeleton dynamics assay, in which cytoskeleton turnover was measured by loss of signal of an actin-interacting fluorescent probe. Whereas an empty vector control lost ~35% of this signal over the time course of this assay, we observed as little as ~10% loss with SAHS4 and preservation of signal to a lesser degree with a number of other IDPs that have turned up positive in other efficacy assays as well (Figure 13). This indicates that these candidates have the ability to slow down the turnover of cytoskeleton components.

Figure 13



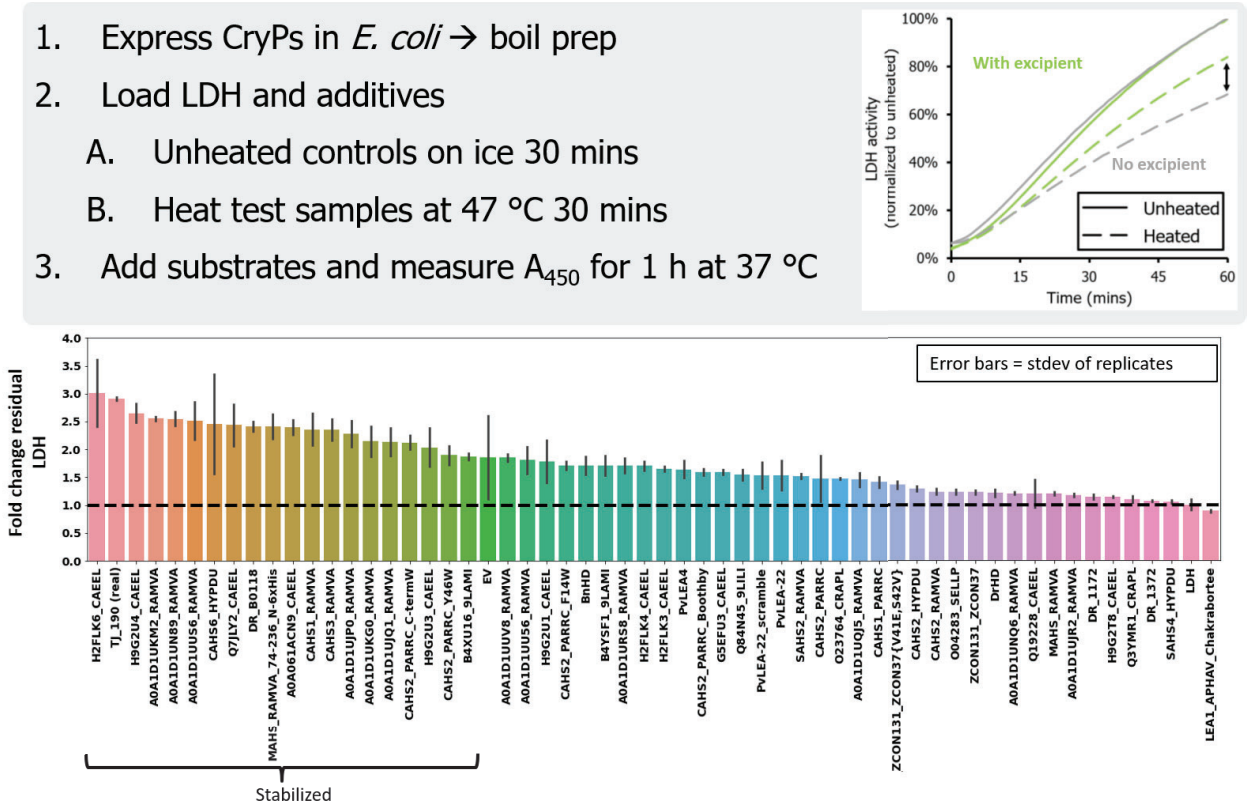
To evaluate the potential for our IDPs to stabilize in a more complex multi-cellular human system, we tested their ability to stabilize the ability of developing liver bud organoids to produce urea, a normal function of hepatocytes (Figure 14). We preliminarily found that DHR81 (TJ_190 in Figure 14) led to 50% increase in urea production after 49 days of development, which is indicative of healthy hepatocytes. Perhaps some of the tardigrade-specific IDPs also had a less significant positive effect. However, as the pursuit of organoid studies was cut by the program soon after we obtained this result, we were unable to follow-up on this result.

Figure 14



As an indicator of how our proteins might stabilize cell-free proteins for improved shelf life or transportability, we tested our candidate IDPs for the ability to stabilize pure lactate dehydrogenase (LDH) enzyme against thermal stress. We expressed and crudely purified IDPs from *E. coli* cultures by boil prep to use in this assay. We found that a high number of IDP samples prepared in this way protected LDH against thermal stress (Figure 15); however, the empty vector control also protected LDH. This is not so surprising as *E. coli* also encodes heat soluble proteins such as heat shock proteins and chaperones that likely could have such protective effects. Notably, a much smaller number of IDP samples showed protective effects above that of the empty vector control. Those included DHR81 (TJ_190 in Figure 15) and multiple LEAs and tardigrade-specific IDPs.

Figure 15



As perhaps a better model of a cell-free protein therapeutic than LDH, we planned to test for the ability of our candidate IDPs to stabilize an approved protein drug, tissue plasminogen activator (TPA) **(Task 1.B.4)**. We established the TPA activity plate reader assay in our lab, but due to pandemic-related shipment delays, we ran out of time before actually getting to test our library for protection of TPA against environmental stress before the end of our performance period.

<u>Milestones by task</u>
2.B.1 In vitro simple human cell culture and preliminary organoid
Milestone: Stasis tested in ≥1 human cell line and 1 human organoid.
Progress: Tested stasis in HEK293s, HT-1080s, BJ5tas, IPSC spheroids, and liver bud organoids.

2.C.1 In vitro stasis efficacy assays
Milestone: 5 in vitro stasis assays and performance data for IDPs.
Progress: Milestone achieved.

1.B.4 In vitro stabilization assays
Milestone: At least 1 IDP that increases function of an approved protein pharmaceutical relative to untreated sample following stress condition.

Progress: Although we demonstrated multiple IDPs capable of stabilizing cell-free LDH enzyme and tested the activity assay of the approved protein drug TPA, we did not get to test IDPs for stabilization of TPA due to long shipping delays for reagents for the TPA assay.

2.C.2 Stasis efficacy assays
Milestone: 12 stasis efficacy assays for complex systems and performance data.
Progress: Milestone achieved.

Characterization of untargeted effects of candidate biostasis IDPs

In addition to the specific functional assays described above, we characterized the broader untargeted effects of 3 chosen diverse IDPs (CAHS2, APOE_80-255, and PvLEA-22) by multi-omics measurements **(Task 2.D.1)**. We measured transcriptomics, proteomics, and metabolomics for human cells expressing these proteins compared to empty vector and GFP-expressing controls. Very few significant differences in transcription were found with expression of our candidate IDPs (Table 1). These included a particular NFkB2 spliceoform increase in PvLEA-22 samples that may explain the activation phenotype we observed for this protein in our NFkB assay; total NFkB2 transcripts were not significantly different across samples, however. Other transcripts that showed increases in these IDP-expressing cells were from genes involved in general transcription, translation, development, and cell morphology.

Table 1

Symbol	Name	CryP	Read Count	Fold Change	p-value (raw)
NFKB2	Nuclear factor NF-kappa-B p100 subunit	PvLEA-22	2180	18.8	0.0086
CASP6	Caspase-6	PvLEA-22	1199	10.2	0.0020
EIF4A2	Eukaryotic initiation factor 4A-II	PvLEA-22	5402	7.4	0.0085
EIF4G1	Eukaryotic translation initiation factor 4 gamma 1	PvLEA-22	2102	7.3	0.0081
GTPBP3	tRNA modification GTPase GTPBP3, mitochondrial	PvLEA-22	3196	6.6	0.0044
CD81	CD81 antigen	PvLEA-22	5254	6.4	0.0054
ZNF615	Zinc finger protein 615	PvLEA-22	1383	5.6	0.0009
NHS	Nance-Horan syndrome protein	PvLEA-22	1438	5.6	0.0087
SRSF2	Serine/arginine-rich splicing factor 2	PvLEA-22	3113	5.1	0.0038
DDOST	Dolichyl-diphosphooligosaccharide--protein glycosyltransferase 48 kDa subunit	CAHS2	8120	13.6	0.0015
CELF2	CUGBP Elav-like family member 2	CAHS2	1953	2.8	0.0016
TCTN3	Tectonic-3	ApoE	3149	10	0.0001
EIF4A2	Eukaryotic initiation factor 4A-II	ApoE	5402	8.6	0.0044
ZFP62	Zinc finger protein 62 homolog	ApoE	1254	7.5	0.0095
CD81	CD81 antigen	ApoE	5254	6.5	0.0031
CEP44	Centrosomal protein of 44 kDa	ApoE	2209	5.2	0.0084

To complement the transcriptomics measurements, we also performed proteomics on the same samples (Table 2). Only one protein was significantly upregulated (p-value < 0.01) in our samples, and only in the CAHS2-expressing cells; this protein was cytochrome P450 1B1 (CYP1B1), which is known to detoxify xenobiotics, including camptothecin (the compound used to induce apoptosis in our apoptosis assay). CYP1B1 was upregulated 23-fold with CAHS2 expression but not transcriptionally. This observation could explain why CAHS2-expressing cells are resistant to camptothecin treatment in our apoptosis assay results, but CYP1B1 is not upregulated with expression of PvLEA-22 or APOE, both of which also reduce apoptosis and to a greater degree. So this hypothesis about CYP1B1 function may not extend to other IDPs we tested. Nothing passed the significance filter in our metabolomics measurements; however, succinyl-CoA increased >2-fold (raw p-value < 0.05) in all IDP-expressing samples. This result could indicate increased TCA cycle activity, which in turn could be caused by high protein catabolism in response to overexpression of IDPs.

Table 2

Symbol	Name	CryP	Fold Change	p-value (raw)
NCBP2AS2	(induced by hypoxia, promotes tumor angiogenesis)	PvLEA-22	2.08	0.09
ESRRB	Steroid hormone receptor ERR2	PvLEA-22	2.67	0.06
RBM5	RNA-binding protein 5	PvLEA-22	2.16	0.02
CYP1B1	Cytochrome P450 1B1	CAHS2	23.39	only seen in 1/3 controls
CCDC158	Coiled-coil domain-containing protein 158	CAHS2	12.24	0.09
ARHGAP31	Rho GTPase-activating protein 31	ApoE	8.02	0.03
LYSMD2	LysM and putative peptidoglycan-binding domain-containing protein 2	ApoE	4.07	0.04
LAMA3	Laminin subunit alpha-3	ApoE	2.71	0.06
IFT81	Intraflagellar transport protein 81 homolog	ApoE	2.47	0
ESRRB	Steroid hormone receptor ERR2	ApoE	2.4	0

In addition to the untargeted multi-omics measurements, for one test case (CAHS2) we also performed immunoprecipitation coupled to mass spectrometry (IP-MS) to detect any potential human proteins that might interact with CAHS2 **(Task 2.D.1)**. Very few proteins showed any degree of enrichment in our CAHS2-expressing samples relative to negative controls and those that did were very slight (Table 3). These proteins function in regulating apoptosis, mitochondrial function, stress response, signal transduction, and protein trafficking. Six of these ten proteins are highly conserved in tardigrades and may bind similarly to CAHS2 in their native context, although again, these enrichments are quite fairly small.

Table 3

Accession	Description	Fold change	Location
P05141	ADP/ATP translocase 2	5.42	mitochondria
Q99714	3-hydroxyacyl-CoA dehydrogenase type-2	1.74	mitochondria
P38646	Stress-70 protein, mitochondrial	1.74	mitochondria
Q15942	Zyxin	1.87	cytoskeleton
O14950	Myosin regulatory light chain 12B	1.76	cytoskeleton
P16949	Stathmin	1.55	cytoskeleton
P32119	Peroxiredoxin-2	1.57	cytoplasm
P98179	RNA-binding protein 3	1.64	cytoplasm
P22090	40S ribosomal protein S4, Y isoform 1	1.59	cytoplasm
Q9P2R3	Rabankyrin-5	2.52	cytoplasm

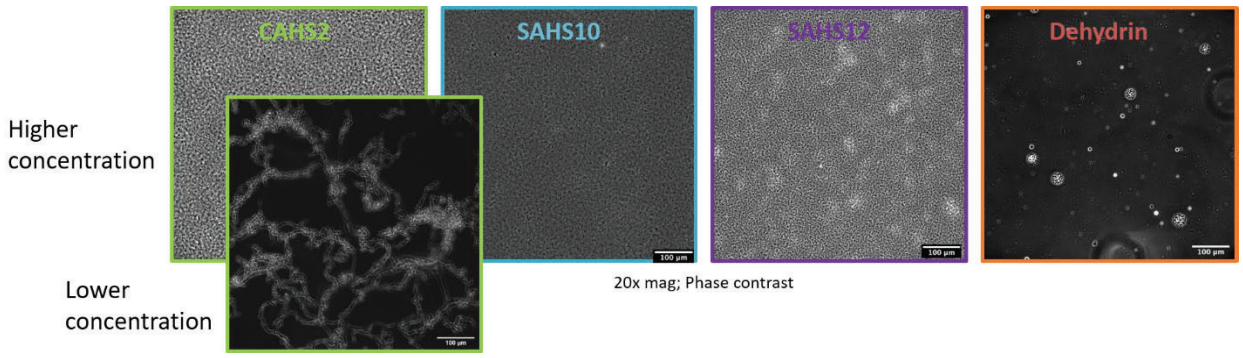
Milestones by task

2.D.1 Impact of candidate IDPs on proteome, metabolome, and transcriptome (cells, organoids)
Milestone: Performance data for IDPs in ≥1 cell types and including 1 organoid. Proteomics, metabolomics, and transcriptomics for at least 3 unrelated IDPs and initial binding partner data for ≥1 IDP in ≥1 cell type.
Progress: We completed multi-omics measurements for 1 cell type and 3 unrelated IDPs and binding partner data for 1 IDP; however we did not generate this data for organoid samples.

Some IDPs protect cells by way of forming phase-separated condensates

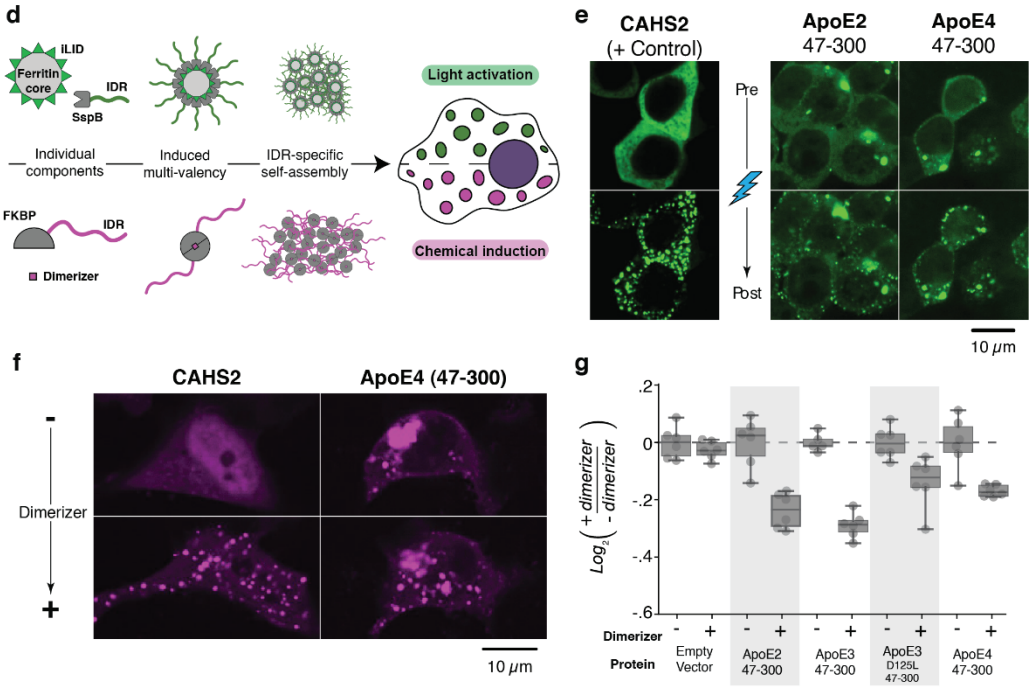
In our early efforts, we found that crudely purified samples of diverse IDPs formed a gel-like material or otherwise liquid droplets *in vitro* by phase-contrast microscopy (Figure 16). This was consistent with similar anecdotal evidence for gelation or “vitrification” suggested by previously published work. In subsequent screens for phase-separation or higher-order structure formation in general by NanoDSF and UV-vis spectroscopy, we confirmed that these and several other candidate biostasis IDPs formed phase-separated structures and that this property did not require dehydration **(Tasks 1.B.2)**.

Figure 16



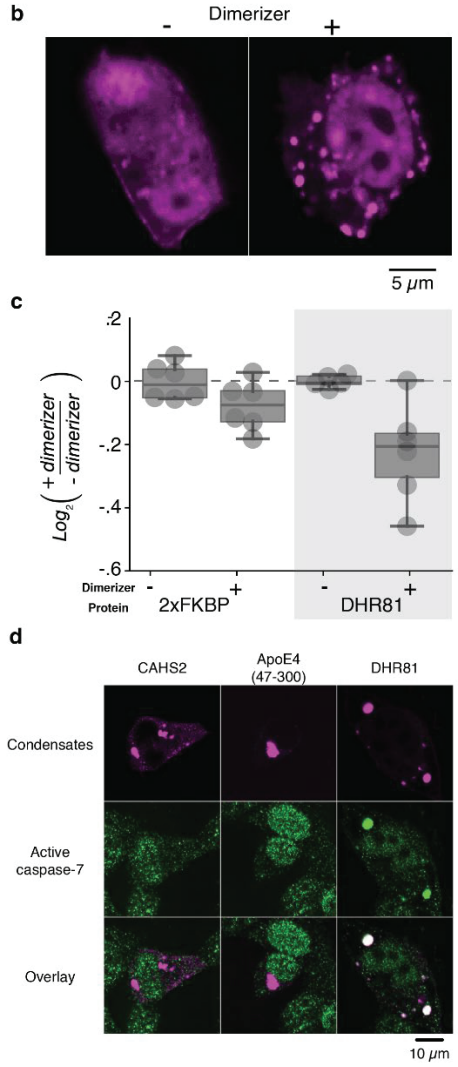
Furthermore, this finding directed us to forego design efforts for optimization of water replacement by IDPs subsequently **(Task 1.A.7)**. Instead, we strategized to evaluate whether candidate IDPs also formed higher-order structures in the cellular environment and, furthermore, if this property was part of the stabilizing mechanism observed for these proteins in our efficacy assays. Several of our top-performing IDPs in the apoptosis assay were observed to form phase-separated condensates when expressed in human cells by fluorescence microscopy in which the IDPs were FLAG-tagged and used an anti-FLAG-FP fusion to visualize them. Recognizing that this could still be simply a correlative relationship, we used a synthetic biology strategy to controllably induce the formation of these condensates by fusing IDPs to light- or chemically-inducible multimerization domains (Figure 17d), the induction of which indeed kinetically promoted condensate formation above and beyond what the IDPs alone did (Figure 17e-f and Figure 18b) **(Task 1.B.5)**. Testing some of these inducible fusions within our apoptosis assay, we were able to show that induction of condensate formation of APOE variants (Figure 17g) and DHR81 (Figure 18c) directly led to anti-apoptotic activity of these IDPs.

Figure 17



Finally, as a preliminary characterization of the specificity versus non-specificity of these protective IDPs for subcellular targets, we performed co-localization analysis of these condensates with active Caspase-3/7 by microscopy. While we found no significant co-localization of Caspases with other IDPs, we found highly significant co-localization of Caspase-7 with DHR81 (Figure 18d). This result suggests that at least DHR81 is directly interacting with and sequestering Caspase-7 into the phase-separated condensates that it forms in human cells, thereby restricting its apoptotic pathway activity. It is still possible that other anti-apoptotic IDPs in our library may sequester other pathway components or may have broad, non-specific stabilizing effects such as generally slowing down cytosolic diffusion.

Figure 18



<u>*Milestones by task*</u>

1.B.2 Stasis induction under hydration

Milestone: Decide to (a) Eliminate dehydration as requirment for stasis and/or (b) focus IDP design principles on hydrated functionality.

Progress: All of our assays supported that stabilization and even higher-order structure formation was achievable under hydration. Thus, we eliminated dehydration as a requirement.

1.A.7 Water replacement optimization

Milestone: Software design tool for IDP water replacement.

Progress: Because the outcome of 1.B.2 was that we determined dehydration was not required for stabilization by IDPs, it was not necessary to develop a design tool for water replacement.

1.B.5 Deeper characterization of proteins that phase-separate in vitro

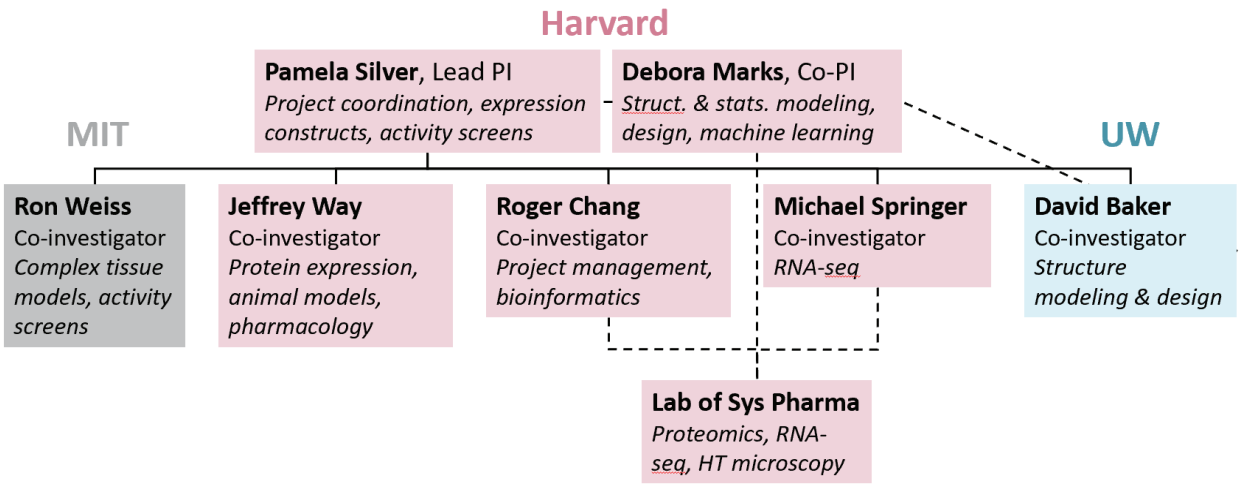
Milestone: Quantitative data for ≥10 candidate biostasis IDPs found to phase-separate and/or yielding positive results in cell-based assays.
Progress: Milestone achieved. We also developed additional systems for generic control of phase-separating IDP condensates in cells, including mixed-population IDP condensates, layered-phase-separated condensate shells, sequestration of target cargo proteins, and surfactant fusion IDPs intended to disrupt formation of condensates by naturally occurring IDPs. This is above and beyond what was proposed for this subtask.

4 Project Coordination, Dissemination, and Translation Efforts

4.1 Project Coordination

During the performance period for this DARPA-supported work, we have coordinated our team with a hierarchical management structure, multiple regularly scheduled sub-group meetings, and an organized central file repository.

In our hierarchical management structure (see figure below), each Co-PI/I has directly managed the day-to-day efforts of postdocs, students, and technicians working under them. Our project manager Roger Chang has coordinated among the collaborative groups and scheduled ad-hoc meetings outside of the regular schedule as needed. All reporting was coordinated by Roger, who liaised with DARPA throughout. We saw significant turnover of research-level personnel over the performance period, and recruited as necessary to fill necessary roles on our team.



We maintained two consistent biweekly subgroup meetings throughout the performance period: 1) experimental sub-group and 2) computational sub-group. All personnel attended one or both of these subgroups depending on their role on the project. These meetings consisted of technical research updates with prepared presentation slides, troubleshooting, sometimes live

analysis, and planning for the next two-week period; all attending personnel had these items discussed at each meeting. Remote collaborators attended these meetings by Zoom. The project manager re-reviewed the minutes and slides from these meetings before preparing each 6-week technical report.

We maintained a central repository for data, presentations, and other files of interest on Dropbox that was shared among all personnel across all participating institutions. The project manager also took responsibility for organizing this repository and managing access.

4.2 Dissemination and Translation (if applicable)

As a result of this DARPA-supported effort, the Silver lab began collaborating with Chris Mason's lab at Weill Cornell on studying the functionality of expressing the tardigrade DSUP protein in human cells. This collaboration has gone beyond the level of characterization we had originally planned for the DARPA-supported effort and included detailed multi-omics analysis focused on genome-binding regions, chromatin formation, and transcriptional activities affected by expression of DSUP. We are currently preparing a manuscript presenting this work.

5 Publications and Presentations

Title, Authors	Description/Type	Date Sent to DARPA/Agent	Status
Engineering Radioprotective Human Cells Using the Tardigrade Damage Suppressor Protein, DSUP, Craig Westover, Deena Najjar, Cem Meydan, Kirill Grigorev, Mike Veling, Sonia Iosim, Rafael Colon, Sherry Yang, Uriel Restrepo, Christopher Chin, Daniel Butler, Chris Moszary, Savlatjaton Rahmatulloev, Ebrahim Afshinnekoo, Roger Chang, Pamela Silver, Christopher Mason	Preprint, bioRxiv	11/10/20	Published
Natural and designed proteins inspired by extremotolerant organisms can form condensates and attenuate apoptosis in human cells, Mike Veling, Dan Nguyen, Nicole Thadani, Michela Oster, Nathan Rollins, Kelly Brock, Neville Bethel, David Baker, Jeffrey Way, Debora Marks, Roger Chang, Pamela Silver	Preprint, bioRxiv	10/1/21	Published

Natural and designed proteins inspired by extremotolerant organisms can form condensates and attenuate apoptosis in human cells, Michael Veling, Dan Nguyen, Nicole Thadani, Michela Oster, Nathan Rollins, Kelly Brock, Neville Bethel, Samuel Lim, David Baker, Jeffrey Way, Debora Marks, Roger Chang, Pamela Silver	Paper, ACS Synthetic Biology	11/10/21	Published
A Comprehensive Multi-omic Mapping of Radiotolerant Dsup-Expressing Human Cells Reveals Chromatin Architectural Protein like Properties, Craig Westover, Deena Najjar, Cem Meydan, Kirill Grigorev, Mike Veling, Roger Chang, Rafael Colon, Sonia Iosim, Sherry Yang, Christopher Chin, Daniel Butler, Christopher Moszary, Ebrahim Afshinnekoo, Pamela Silver, Christopher Mason	Paper	Preparing now	Preparing now
Anti-apoptotic condensates of extremotolerance-related proteins, Dan Nguyen, Mike Veling, Nicole Thadani, Michela Oster, Nathan Rollins, Kelly Brock, Neville Bethel, David Baker, Jeffrey Way, Debora Marks, Roger Chang, Pamela Silver	Presentation, American Physical Society	3/18/22	Preparing now