Natural and designed proteins inspired by extremotolerant organisms can form condensates and attenuate apoptosis in human cells

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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 driving protective proteins into the condensate state increases apoptosis protection, and highlight the ability for DHR81 condensates to sequester caspase-7. Taken together, this work draws a link between extremotolerance-associated proteins, condensate formation, and human cellular protection.

INTRODUCTION

When exposed to extreme environmental stress, extremotolerance-associated (ExTol) organisms enter a state of reduced cellular metabolic activity that promotes survival in harsh conditions. This state has been shown to protect these organisms from high salinity, desiccation, and even the vacuum of outer space (Jonsson et al., 2008). To acclimate to
these conditions, *ExTol* organisms significantly upregulate the production of protective metabolites, like trehalose (Neuman, 2006), as well as tolerance-conferring proteins (Boothby et al., 2017), many of which are intrinsically disordered proteins (*IDPs*) with repetitive sequence and predicted amphipathic helices.

While the mechanism of protection is unknown, one model suggests these proteins mediate formation of a gel- or glass-like material state within an entire cell to protect it from damage (Boothby et al., 2017; Hengherr et al., 2008). The model posits that such proteins promiscuously interact with membranes and proteins, increasing intracellular viscosity and protecting vulnerable structures (Hand et al., 2011) (Figure 1A Top). Such interactions would also work to prevent stress-induced protein unfolding and protein aggregation (Chakrabortee et al., 2010). An alternative model suggests that these proteins form intracellular phase-separated membraneless compartments with distinct physical parameters (referred to here as *condensates*) that partition key stress-sensitive cellular components (Belott et al., 2020) (Figure 1A Bottom). Within these dense protein structures, essential components are sequestered and protected from damage. The models are not mutually exclusive; for example, in the case of desiccation tolerance, continued dehydration could lead phase-separated compartments to fuse, comprise much (if not all) of the cell, and promote a glassy material state.

The resurgence of interest in biological phase separation, extensively reviewed in (Boeynaems et al., 2018; Choi et al., 2020; Hyman et al., 2014; Shin and Brangwynne, 2017), has shown that human cells can employ similar strategies to survive stressful
conditions. While the reversible formation of stress granules in healthy cells is the archetype of stress tolerance, recent studies have revealed phase separation as a key adaptation that cancer cells use to avoid apoptosis and cell death (Jiang et al., 2020). Phase-separated condensates have been implicated in sequestering key tumor repressors (Jiang et al., 2020), enhancing or repressing gene expression in response to stress (Boija et al., 2018), and concentrating and protecting key metabolites like nucleotides (Hubstenberger et al., 2017). These processes parallel the mechanisms that ExTol proteins are thought to use to protect complex biological systems, suggesting there could be some functional overlap. That is, sequence features and properties within ExTol proteins could be implicated in mammalian stress tolerance pathways, including apoptosis resistance.

A key sequence feature of some ExTol proteins is the presence of amphipathic helices (Tolleter et al., 2007). While disordered in most contexts, ExTol proteins can transiently form amphipathic helices in certain molecular environments, notably when interacting with and stabilizing membranes (Hand et al., 2011). The helices often exhibit a hydrophobic face, which interacts with core lipids within the membrane, flanked by positively charged residues that might interact with the negatively charged lipid head groups (Figure 1B). This amphipathic moment often repeats across the length of the protein (Figure 1C). This feature is a hallmark of the late embryonic abundant (LEA) class of ExTol proteins (Hand et al., 2011). Human membrane interacting proteins, like alpha-synuclein, also exhibit a similar repeat motif (Bussell and Eliezer, 2003). Such parallels in sequence features
suggest that orthogonal expression of ExTol proteins may confer their protective properties to other biological systems, like human cells.

Indeed, previous reports have shown that some of these ExTol proteins provide similar protective properties when expressed heterologously. Expressing tardigrade-specific IDPs (TDPs) in bacteria provides protection against desiccation (Boothby et al., 2017). Anhydrin, an IDP upregulated by *Aphelenchus avenae* during dehydration, expressed in a human cell line limited PABPN1-A17 aggregation, a hallmark of oculopharyngeal muscular dystrophy (Chakrabortee et al., 2010). Similar reports demonstrated protection of human cells provided by expression of LEA proteins originating from the African midge (Li et al., 2012) and by TDPs from tardigrades (Tanaka et al., 2015). These findings reinforce the promise of transferring protective phenotypes from their native contexts to human cells.

Taken together, these data indicate a potential avenue for using this class of proteins to protect biological systems in a controlled manner. To test this hypothesis, we generated approximately 300 sequences inspired by ExTol proteins and examined their ability to protect against apoptotic cell death. Our results show that human proteins containing sequence hallmarks of ExTol proteins as well as *de-novo* designed condensate forming proteins can protect human cells from chemically-induced apoptosis. We also demonstrate that condensate induction using synthetic modifications can strengthen apoptotic protection. Finally, we show that key components of the apoptotic pathway can
localize to inducible condensates, suggesting enzyme sequestration could be a mechanism of action.

RESULTS

Assembling a library of stress tolerance proteins

Based on the hallmark sequence features of ExTol proteins, we searched the published literature to identify proteins that not only contain these features but also exhibit some evidence for protection of biological structures. The library was generated based on over one hundred naturally-occurring proteins based on five lines of evidence for stress tolerance association, including: change in expression during extremotolerance, knockdown or overexpression changing the tolerant state, *in vitro* protection, orthogonal expression transferring tolerance, and sequence similarity to other proteins with these features (Figure 1D and Supplemental table 1).

A PFAM domain analysis of the identified proteins reveals almost half of the identified targets have two key domains: Late Embryonic Abundant (LEA) and Apolipoprotein E (ApoE) domains (Figure 1E, Supplemental table 1). LEA proteins are well known to be upregulated during the last stages of plant seed maturation (Mertens et al., 2018). This domain is thought to protect the seed embryo during transport to its new destination. There is also evidence that this domain has been co-opted by bacteria as protection from desiccation and radiation (Makarova et al., 2001). ApoE proteins interact with lipid particles to allow for lipid reuptake into cells. Additionally, mutations within human ApoE have been identified as strong genetic predictors of Alzheimer's disease in human
populations (Yamazaki et al., 2019). Based on their lipid interaction properties and potential role in neuronal homeostasis, as well as its prevalence in the PFAM domain analysis, we decided to include human ApoE and its various mutations in our study as an ExTol protein.

**Designing novel extremotolerance-associated proteins**

To test hypotheses about sequence features and their relationship to phase separation and apoptosis, we designed novel proteins based on the set of ExTol proteins. These new designs take 3 approaches (Figure 2): truncation of proteins to focus on repetitive, intrinsically disordered, and amphipathic regions; isolation of individual helices with desired sequence features; and *de novo* design of alpha helix-containing proteins that self-assemble into higher order structures. Through these designs, we sought to identify the minimal elements required for protection and phase separation.

First, we identified regions within natural proteins that contain intrinsic disorder (Erdos and Dosztanyi, 2020), repetitive sequences (Biegert and Soding, 2008), and amphipathic helices (Gimenez-Andres et al., 2018; Segrest et al., 1992). Figure 2A illustrates the use of HHRepID on the sequence of full length human ApoE. Based on this analysis, we identified the repeating domain within ApoE for further testing. Other ExTol proteins were similarly modified and examined.

We also included short peptides based on the consensus sequence of repetitive regions that have previously been shown to be protective (Furuki et al., 2019). To test if any of
the sequence features we identified are important for apoptosis protection, we altered these features within these short peptides. For example, we altered the amphipathic character of these helices (Figure 2B) to determine if apoptosis protection was changed.

To examine whether generic condensate formation is sufficient to confer apoptosis protection, we also examined completely synthetic alpha helix-containing proteins that were designed to form repetitive structure with sequences orthogonal to naturally-occurring proteins (Brunette et al., 2015). These designed helical repeat (DHR) proteins interact along the axis of the helix, forming long spiraling fibrils, but may also potentially self-interact along the face of the protein based on a pattern of positive and negative charges (Figure 2C). This ability to oligomerize and form multivalent complexes, which may then interact via weak non-covalent interactions, is an established determinant of condensate formation (Choi et al., 2020).

Together, our library consists of 104 naturally-occurring proteins, 166 truncations or isolations of helical regions, and 19 de novo designs (Figure 2D). Taken altogether, these proteins interrogate several hypotheses about how disordered proteins may protect human cells against apoptosis.

Apoptosis assay identifies extremotolerance-associated proteins in human cells

To model stress in a mammalian cell system, we induced cellular apoptosis by treatment with the chemotherapeutic camptothecin (CPT), a DNA topoisomerase inhibitor well known to trigger apoptosis (Li et al., 2017; Wall et al., 1966). Under these conditions,
protection against apoptosis could be achieved through a number of avenues, including protection against the DNA damage caused by CPT-induced topoisomerase inhibition (Li et al., 2017), interruption of the signaling cascade, or even protection of the mitochondrial membrane whose rupture ultimately triggers apoptosis (Bortner and Cidlowski, 2002).

To test our designs for protection against apoptosis, we used a human cell-based apoptosis assay (Figure 3A). This assay uses HT-1080 fibrosarcoma cells from ATCC (Rasheed et al., 1974). These cells are seeded in a 96-well plate and allowed to adhere overnight. The next day, the cells are co-transfected with a plasmid containing the ExTol candidate proteins of interest as well as a transfection marker. The following day, the media is swapped to one containing 13.2 µM CPT. After addition of CPT, caspase-3 and -7 activation is measured by microscopy every 45 minutes for 1 day using a fluorophore quencher pair attached to either end of a peptide with the sequence “DEVD”. As caspase-3 and -7 are activated, the DEVD peptide is cleaved, releasing the fluorophore from the quencher. The unquenched fluorophore localizes to the nucleus where it can be quantified by imaging on a single-cell level. The single-cell resolution allows for the separation of transfected and non-transfected cells based on the transfection marker. Only single cells with the transfection marker were subjected to further analysis; Figure 3B shows sample images highlighting the type of data being analyzed.

On every plate, an empty vector negative control is run as well as a positive control containing the X-linked inhibitor of apoptosis (XIAP). This protein is known to bind to caspase-3 and -7 to block their activity (Obexer and Ausserlechner, 2014). Figure 3C
shows example data comparing median DEVD peptide signal intensity over time after induction with CPT of four wells on the same plate transfected with XIAP and empty vector. The results show a reduction in DEVD peptide signal intensity when the XIAP-containing vector is transfected, demonstrating that the assay can identify modulators of apoptosis.

To convert these time-resolved data to a measure of protection against apoptosis, the maximum DEVD peptide signal intensity is calculated and normalized to a plate-matched empty vector control (see methods for further details). The normalized apoptosis induction intensity is shown in Figure 3D left. The data are slightly skewed towards apoptosis-protection, which supports our hypothesis that the targeted proteins can protect against apoptosis.

To highlight the top-performing designs, the top 10% of the targets are shown in Figure 3D right. As expected, the XIAP positive control showed the strongest anti-apoptotic effect. The two top-performing hits were ApoE designs. Human alpha-synuclein is the 5th hit, which is expected as it is known to be both intrinsically disordered, and anti-apoptotic (Li and Lee, 2005). Additionally, several ApoE designs and short LEA helices are top hits. The data used to generate these figures is included in supplemental table 2.

**Known extremotolerance-associated proteins affect apoptosis**

Among the top hits in apoptosis reduction are an IGKV protein highly expressed in mucus of the Chinese giant salamander *Andrias davidianus* (Geng et al., 2019) that is used as
a medical glue in traditional Chinese medicine, an LEA protein from the nematode *Aphelenchus avenae* previously shown to have the ability to reduce a form of protein aggregation associated with oculopharyngeal muscular dystrophy in human cells (Chakrabortee et al., 2010), and a mitochondrial LEA protein from the brine shrimp *Artemia franciscana* shown to protect human cells during desiccation (Li et al., 2012) (Figure 3D). The prominence of these ExTol proteins suggests the assay can help identify other proteins that can protect cells from more general stresses beyond CPT-induced apoptosis.

**Short repetitive peptides and de novo designs modulate apoptosis**

LEA repeat-containing peptides are among the top-performing hits in the screen. While the original short LEA designs from Furuki et al. 2019 (Furuki et al., 2019) had a minor effect reducing apoptosis, several of the designs tested here performed better (Supplemental Figure 3A). As 22 amino acids is small and could lead to peptide stability issues, longer 50-amino acid designs inspired by the same LEA proteins Furuki et al. utilized were also developed (Supplemental Figure 3B). Some of these designs were also able to reduce apoptosis, including the PvLEA4 with extended repeats (notably PvLEA4 repeats extended #2), which fell into the top hits of the entire library (Figure 3D and Supplemental Figure 3B). These results suggest that optimization of synthetic designs yields proteins with stronger function than naturally-occurring proteins.

While these proteins were designed to interrogate the relationship between sequence and apoptosis protection in short peptides, little relationship was found with respect to the
sequence features we analyzed (Supplemental Figure 3C). Several designs were made that would alter the hydrophobic moments of putative helices these peptides are predicted to form. To examine if this sequence property relates to the stress-tolerance function of these proteins, we attempted to correlate this property with assay performance. However, no significant correlation was found, suggesting (at least in these cases) that degree of amphipathicity on its own is not the key determinant of stress-tolerance function of these proteins.

**The repeat region of ApoE comprises its minimal anti-apoptotic unit**

The top two hits in the assay were ApoE4 (47-300) and ApoE2 (47-300). These truncated forms of ApoE maintain the repeat domains shown in Figure 4A, while removing the signal peptide and terminal regions. This suggests that the repeat region of ApoE accounts for its core anti-apoptotic functionality. Interestingly, the full length ApoE2 and E4 do not show up as top hits, suggesting that these truncated forms improve on the apoptosis protection provided by the full-length variants (Figure 4B).

Based on the location of the signal peptide, it is expected that ApoE4 should localize to the secretory pathway whereas the truncated form may be cytoplasmic. To test this hypothesis, cells were subjected to Western blot and immunofluorescence analysis using commercially available antibodies (Abcam ab24139; Figure 4C and Supplemental Figure 4A). The Western blot results show that both the full-length and truncated forms of ApoE4 are expressed at the expected size (Supplemental Figure 4A). The immunofluorescence shows co-localization between the ApoE4 full length protein and the endoplasmic
reticulum (marked by Thermo’s PDI antibody MA3-019, Figure 4C). However, the truncated form did not show the same co-localization, suggesting as expected that it may not enter the secretory pathway as the full-length version does.

Strikingly, the truncated version appeared to form more pronounced puncta throughout the cytoplasm of the cell, suggesting the formation of potentially phase-separated condensates. In screening through the point mutants of ApoE also tested here, we found a similar condensate phenotype with the full length ApoE3 D125I mutation (Supplemental Figure 4B). Together, these data suggest that ApoE may be able to support condensate formation and confer apoptosis protection in part via the protective compartment mechanism (Figure 1A bottom).

CAHS2 and ApoE form condensates upon induced multivalency

Established condensate engineering tools (Bracha et al., 2019) were used to test for latent phase separation propensity within the top performing proteins. We used both light- and chemically-inducible systems of multivalency. In the light system, a ferritin core (comprised of self-assembled monomers, FTH1 domain-eGFP-iLID) becomes decorated with SspB-modified proteins upon exposure to blue light (Bracha et al., 2018) (Figure 4D top). In the chemical system, multivalency (i.e. multiple arms of the ExTol protein of interest) is induced using a dimerization agent (Stanton et al., 2018) that brings together FKBP-modified proteins (Bracha et al., 2019) (Figure 4D bottom). These inducible methods increase the valency of a system, and thus work to favor phase separation. In
this way, they can help reveal any latent ability to form condensates that is not immediately observable without modification.

Indeed, condensates were observed in both inducible systems using ExTol proteins. CAHS2, a protein from the tardigrade *P. richtersi*, exhibited the most dramatic phenotype, which is consistent with recent work describing the gel forming capabilities of CAHS proteins (Yagi-Utsumi et al., 2021) (Figure 4E&F). ApoE variants were of particular interest, based on the puncta observed in the top performing ApoE constructs. All versions of ApoE tested formed puncta without activation, consistent with our previous observation using untagged native ApoE (Figure 4C). Upon light- or chemical-activation, there is a noticeable increase in condensates, exemplifying how phase separation can be enhanced with increased multivalency (Figure 4E&F).

**Induction of condensates reduces apoptosis**

Induction of multivalency in ApoE containing constructs reduces apoptosis. We performed the apoptosis assay using the top hits modified for chemically-induced multivalency, namely fusion to two FKBP domains. The results show that the presence of the chemical dimerizer did not affect apoptosis signal in cells transfected with the empty vector; in contrast, the ApoE constructs exhibit reduced apoptosis with added dimerizer (Figure 4G). That is, artificially increasing ApoE condensates was apoptosis protective. These data suggest that condensate formation plays a role in the anti-apoptotic effect of these proteins.
Synthetic DHR81 condensates reduce apoptosis and sequester caspase-7

Induction of DHR81 based condensates protects against apoptosis. DHR81 is formed from four helical hairpin repeat regions; the core of the protein is hydrophobic to ensure consistent folding, while the outside of the protein is decorated with checkerboard positive and negative charges (Figure 5A) (Brunette et al., 2015). This structure ensures the protein is well folded, while also enabling the potential for higher order structure formation via electrostatic interactions. Indeed, as with the ApoE variants, DHR81 supported condensates in the chemically-inducible condensate system (Figure 5B) and exhibited a stronger anti-apoptotic phenotype when multivalency is induced (Figure 5C and Supplemental Figure 5A). There was little to no effect in cells containing an empty vector or expressing the FKBP dimerization domains alone.

To further explore how condensates mediated by ExTol proteins may confer an anti-apoptotic effect, we examined whether they sequester major components of apoptotic signaling, namely caspase-3 and -7. The results showed that activated caspase-7 localized to DHR81 condensates but not CAHS2 or ApoE4 condensates (Figure 5D). Caspase-3, however, does not localize to either DHR81, CAHS2, or ApoE4 condensates (Supplemental Figure 5B). These results suggest sequestration of key apoptotic factors could be a mechanism by which these condensates reduce apoptosis.

Discussion

To broadly evaluate the ability of ExTol proteins to protect orthogonal systems from environmental stress, we studied over one hundred different ExTol proteins. We used
these as a basis to design approximately 300 variants, including truncations and fusion proteins. These designs were evaluated on their ability to protect human HT-1080 cells against CPT-induced apoptosis. The results showed that known ExTol proteins, ApoE variants, and the synthetic DHR81 protein could modulate apoptosis levels when transferred to human cells.

Protection against apoptosis is a key feature of many ExTol proteins. As apoptosis is a common cellular response to stress, the ability to mitigate this response is important in providing cell-autonomous protection in stressful environments. While the mechanism resilient organisms use to survive is still not fully understood, it is becoming increasingly clear that phase separation may play an important role.

ExTol proteins are proposed to form expansive higher order structures (e.g. gels or glassy systems) that reduce the viscosity of the cytoplasm, especially in desiccated states (Boothby et al., 2017). Other reports show that these proteins could mediate formation of protective phase-separated compartments (i.e. condensates), sequestering damage-inducing proteins and/or protecting important targets (Belott et al., 2020). Based on our condensate data, it appears the latter may be at play for proteins like ApoE or DHR81. Notably, in these proteins that already exhibit both anti-apoptotic effects as well as elevated phase separation propensities, increasing the amount of condensates via an engineered inducible system improved protection against apoptosis. This observation suggests that condensates could be a key part of the protective mechanism.
Interestingly, we observed that activated caspase-7 partitions into DHR81 condensates, suggesting caspase sequestration as a potential anti-apoptotic mechanism. Phase-separated condensates and higher order assemblies are already implicated in mediating apoptosis: stress granules inhibit apoptosis by suppressing MAPK pathways (Arimoto et al., 2008); paraspeckles sequester pro-apoptotic tumor suppressors (Jiang et al., 2020); the apoptosome relies on Apaf-1 multimerization, which has been reported to undergo amyloid fibrillation in specific conditions (Rao et al., 2009); and stress-dependent phase separation modulates proteasome formation, whose function affects apoptosis (Gupta et al., 2018; Yasuda et al., 2020). It is interesting to note that we only observe clear caspase-7 partitioning into DHR81 condensates, suggesting the possibility of a specific binding interaction in this case. ApoE condensates may be sequestering other apoptotic factors or using a different mechanism to reduce apoptosis.

It is also possible that these ExTol proteins achieve their anti-apoptotic effect by implementing a more general physical mechanism, namely slowing the intracellular mobility of biological effectors. There are various reports describing how the effective viscosity within the cytoplasm can be increased in response to external stresses (Dijksterhuis et al., 2007; Parry et al., 2014) or mediated by protein composition, specifically intrinsically disordered proteins (Theillet et al., 2014). Notably, a pervasive cell-spanning gel or network is not necessary to achieve such an effect; it is possible that the presence of condensates, coupled with their ability to selectively partition biomolecules (i.e. control what biomolecules can enter and diffuse through them), could broadly slow biomolecular movement. That is, by forcing different proteins to either be
trapped in them or diffuse around them, condensates can decrease protein mobility and perturb e.g. signaling pathways. Our results showing a lack of caspase-7 sequestration by some of our protein condensates could thus support this more general mechanism of condensates perturbing biology.

Condensates are becoming increasingly implicated in disease (Shin and Brangwynne, 2017), particularly when their structural metastability and reversibility is dysregulated (e.g. through mutation). Notably, drugs that block the formation of amyloid fibrils and plaques, which are products of phase separation dysregulation, are promising yet strongly debated (Lalli et al., 2021) therapies for many neurological diseases. As such, our observations of ApoE are particularly salient. We found that expression of a cytoplasmic form of ApoE is protective against apoptosis. In particular, the truncated repeat region of ApoE is sufficient and shows the strongest anti-apoptotic activity of all designs we tested. Others have shown similar effects for exogenously added ApoE-containing liposomes protecting rat neurons against apoptosis (Hayashi et al., 2009), though we show the effect through overexpression of ApoE inside of human cells. When combined with our condensate results and the clinical relevance of ApoE in Alzheimer’s disease (Schellenberg et al., 2000), these observations suggest the anti-apoptotic effect observed here warrants further study, to further elucidate its role in Alzheimer’s disease progression.

Altogether, our work expands upon the understanding of the relationship between condensate formation and cell physiology, demonstrating how condensate accumulation
can be broadly beneficial in reducing apoptosis while also identifying diverse new protein
domains that confer protection.

Author Contributions
M.T.V. and D.T.N. designed and performed experiments, analyzed the data, and wrote
the paper. N.N.T. analyzed the data and wrote the paper. M.E.O provided technical
support. N.J.R., K.B.B., and N.P.B., designed constructs. D.B. and J.C.W. managed the
teams and provided key insight. D.S.M., R.L.C., and P.A.S., designed constructs,
managed teams, and wrote the paper.

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Figure 1: Selection of candidate extremotolerance-associated proteins (a) Illustration of different protective models for cellular stress tolerance. (b) Illustration of amphipathic membrane interaction for rotifer LEA4 protein and Alpha-synuclein, thus highlighting a feature indicative of extremotolerance-associated proteins. (c) Hydrophobic moment analysis of rotifer LEA4, showing the repetitive nature of the amphipathic signature. Highlighted green segment corresponds to the LEA4 helix in (b). (d) Venn diagram of the lines of evidence used to select targets in the final library. Abbreviations: KD, knockdown; OE, overexpression; Δ, change in expression levels. (e) Domain analysis of included targets reveals LEA and ApoE domains in almost half of the targets tested. The other half contains proteins with other PFAM domains (19%) or without any affiliated PFAM entry (32%). Of the proteins lacking a known PFAM annotation, many exhibit repetitive sequence (20% of all targets).
Figure 2: Design based on candidate extremotolerance-associated proteins (a) Truncations were made to isolate regions of repetitive and amphipathic sequence. (b) Small helices were selected and altered based on their hydrophobic moment. (c) Synthetic alpha helix-containing proteins designed to oligomerize. (d) Venn diagram highlighting the library that was tested.
**Figure 3:** Screen for CPT resistance by extremotolerance-associated proteins (a)

Flow diagram of the assay. (b) Representative images showing cells during the assay. Cells are identified using SiR-Hoechst marking the nucleus (blue). Transfected cells are identified by the presence of the mCherry marker (green). Finally, the degree of apoptosis is identified by the presence of the cleaved DEVD peptide (red). (c) Positive control showing apoptosis reduction upon expression of XIAP. (d) Bar graph highlighting the
apoptosis resistance provided by different constructs, including zoomed in area to
highlight the top hits in the assay. Source species of a sequence are abbreviated and
shown in italics; abbreviations are drawn from Uniprot, including RAMVA (*Ramazzottius
varieornatus*), HYPDU (*Hypsibius dujardini*), PARRC (*Paramacrobiotus richtersi*),
ANDDA (*Andrias davidianus*), APHAV (*Aphelenchus avenae*), ARTSF (*Artemia
franciscana*), CRAPL (*Craterostigma plantagineum*), and DEIRA (*Deinococcus
radiodurans*).
Fig. 4. ApoE truncations and synthetic condensates further reduce apoptosis. (a) Gene diagram highlighting the repetitive region of ApoE. (b) Boxplots highlighting the apoptosis assay performance of the full length and truncated forms of ApoE2 and E4. (c) Immunofluorescence of ApoE4, full length and truncated forms. White indicates colocalization, which is more apparent with the full-length protein. The truncated form appears to exhibit larger and more distinguishable puncta. (d) Diagram of the induced multi-valency systems used here, including the light-inducible system (top) and the chemically-inducible system (bottom). (e) Condensate formation assay using the light-inducible system, including the CAHS2 positive control showing condensates only upon light induction and several ApoE examples that form condensates both before and after induction. (f) Condensate formation assay using the chemically-inducible system, including the CAHS2 positive control showing condensates only upon added dimerizer and ApoE4 (47-300) exhibiting condensates both before and after induction. (g)
Apoptosis data showing reduction in apoptosis upon condensate induction with dimerizer.

Data are normalized to mean apoptosis level of uninduced (i.e. no dimerizer) sample for each construct.
**Fig. 5. DHR81 condensate formation and apoptosis reduction.** (a) Diagram of DHR81, illustrating a charge distribution that can support oligomerization. (b) Sample images highlighting how the synthetic protein DHR81 supports chemically-induced condensates. (c) Apoptosis is further reduced in the presence of dimerizer (i.e. with induced DHR81 condensates). The FKBP-modifications alone, which enable chemically-induced multi-valency, have a minimal effect on apoptosis. (d) Activated caspase-7 (green) co-localizes with DHR81 condensates but not CAHS2 or ApoE4 (47-300) condensates (magenta). White indicates colocalization.
Figure 3 Supplement (a) Graph and sequences of various LEA repeats tested, with full sequences and performance. (b) Graph and sequences of extended LEA repeats tested.
Correlation analysis for relationship between hydrophobic moment with apoptosis assay performance.
Figure 4 Supplement (a) Western blot data of some ApoE constructs showing expression at the expected molecular weight (b) Sample images showing condensates forming in the full length ApoE3 D125I mutation.
**Figure 5 Supplement** (a) DHR81 inducible condensates reduce apoptosis. (b) Sample images showing no co-localization between activated caspase 3 and condensates.