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Effect of Tardigrade Protein CAHS3 on Stress Tolerance in MDCK Cells

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ABSTRACT OF THE THESIS

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Tardigrades are microscopic eukaryotic organisms with the unique ability to withstand a wide range of hostile conditions including sudden pressure changes, freezing, radiation exposure and desiccation. Their ability to survive in harsh conditions is under active research but the expression of Tardigrade-specific Intrinsically Disordered Proteins (TDPs) are thought to play a role. TDPs are thought to have a chaperone-like role in maintaining protein structure under stress, especially during hyperosmotic and desiccating conditions. It is currently poorly understood the extent to which TDPs offer similar stress tolerance in mammalian cells. The goal of this investigation is to determine the impacts of TDP expression in MDCK cells, a common epithelial cell model. Specifically, we expressed the TDP CAHS3 in MDCK cells and quantified the epithelial phenotype, heat stress tolerance, and solvent stress tolerance. CAHS3 expression was associated with increased stress tolerance in MDCK cells, and protein expression associated with loss of epithelial phenotype.

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1. Introduction

Biological organisms possess a remarkable ability to adapt to a wide range of stressors and living conditions. This is exemplified in tardigrades, microscopic organisms that can withstand some of the most extreme terrestrial stressors, including survival in a completely desiccated state, termed anhydrobiosis. While in this state, tardigrades have been shown to survive exposure to 90 °C, -196 °C, exposure to 4000 Gy of radiation, and 99.8% acetonitrile; conditions that are lethal to most forms of multicellular life, are withstood by the tardigrade.[1]

The mechanisms underlying the tardigrade's robust survival remains incompletely understood, however a few hypotheses have been forwarded. For example, it has been suggested the sugar trehalose could provide this resistance. Trehalose is found in several bacteria and is regarded as a versatile chaperone for increasing desiccation tolerance in *Saccharomyces cerevisiae* and even used to preserve human eggs and sperm[2–4]. However, recent studies investigating trehalose in tardigrades have found low levels of trehalose that did not change during a hydrated vs dehydrated state in certain species of tardigrades while also finding that trehalose was completely absent in another species of tardigrade during desiccation.[5] Still other theories have been investigated such as identifying late embryogenesis abundant (LEA) proteins, a common class of proteins in anhydrobiotic organisms.[6] While, LEA proteins have been discovered in tardigrade species, studies have found that a LEA-like protein class, termed tardigrade-specific intrinsically disordered proteins (TDPs), are chiefly responsible for the tardigrade's ability to withstand

desiccation.[7] Two classes of which, cytosolic abundant heat soluble (CAHS) and secretory abundant heat soluble (SAHS) proteins, are conserved among all tardigrades and unique to their phyla.[8] Furthermore, when either SAHS or CAHS is targeted via RNAi, tardigrade survival under desiccated conditions drops dramatically, with up to 90% decrease in tardigrade viability under anhydrobiotic conditions.[7] Although the exact mechanism of how these unique TDP proteins protect other proteins hasn't been fully elucidated, it is suspected that they might function similar to LEA proteins, which form a "molecular shield" that stabilizes proteins during desiccation, a process similar to but distinct from classic protein chaperones.[6,9]

Protein misfolding and subsequent aggregation is a common result of multiple forms of cellular stress, including oxidative, heavy metal, osmotic, and heat stress. Regardless of the cause, the aggregation of proteins interrupts critical pathways resulting in cell death.[10] Misfolded protein aggregations are typically degraded, repaired or cleared through asymmetric sequestering of built up damage through juxtanuclear quality control (JUNQ) compartments, however rapidly aged of cells accumulate sufficient damage to point where they are incapable of clearing said damage which results in either apoptosis or senescence. [11–14] Senescence is a broad term that contains heterogenous characteristics that are dependent on the method of induction but generally can be described with over expression of certain proteins and a change in cell size. In the instance of heat shock induced senescence, molecular chaperones of the heat shock protein (HSP) family are upregulated to transport/sort proteins and prevent misfolded protein aggregation.[15,16] An upregulation in HSP90 for example, helps to stabilize p21, which in turn triggers cell cycle

arrest and the onset of senescence.[11–14] However, another response to heat shock seen days after is the nuclear localization of YAP which further drives cells to a senescent pathway versus that of apoptosis by stimulating the expression of the anti-apoptotic protein survivin.[17–19] The inhibition of HSP90 can lead to reduced levels of LATS1, a kinase that relays antiproliferative signals in the Hippo tumor suppressor pathway, which leads to decreased phosphorylation of its substrate, YAP.[19,20] Dephosphorylated YAP is then consequently driven into the nucleus which leads to senescence as mentioned before.[21]

While LEA proteins and LEA-like proteins, such as those in HSP family, are known to prevent protein aggregation, relatively untested is how the LEA-like proteins of TDPs might function to increase the stress tolerance of mammalian cells in other contexts, for example, against heat stress, chemical modification, and protein aggregation in instances where senescence is induced.[23]

The application of TDPs in mammalian cells to increase overall cell hardiness would have several implications to biological science, tissue engineering and medicine in the form of durable tissue constructs, as a preservative for biological materials and to create robust cells out of sensitive primary cells. In this paper, we sought to demonstrate further applications of TDP expression in mammalian cells, specifically the expression of CAHS3 in MDCK cells. We analyzed the effects of CAHS3 expression in MDCK cells and the efficacy of TDPs to mitigate the toxic effects of two organic solvents, withstand desiccation, and reduce senescent associated responses during heat shock.

2.0 Methods

2.1 Culture of MDCK cells

Madin-Darby Canine Kidney cells (MDCK; ATCC, Manassas, VA) were cultured in DMEM (Corning, Tewksbury, MA) media containing 3% Fetal Bovine Serum (FBS; Corning), 1% penicillin/streptomycin (P/S; Genesee Scientific, El Cajon, CA) and 0.5 μg/mL of amphotericin. All cells were maintained at 37 °C and 5% CO_2.

2.2 Transfection

Three transfections were carried out to generate three cell types for the purposes of this paper. One expressing the protein of interest, CAHS3, conjugated to the fluorophore AcGFP1, and the other as a control expressing AcGFP1. *E. Coli* stab cultures containing CAHS3 (pAcGFP1-N1-CAHS3 was a gift from Takekazu Kunieda; Addgene plasmid #90031; http://n2t.net/addgene:90031; RRID:Addgene_90031) and AcGFP1 (AcGFP1-N1 was a gift from Michael Davidson; Addgene plasmid #54705; http://n2t.net/addgene:54705; RRID:Addgene_54705) were purchased from AddGene (Addgene, Watertown, MA). 50 mL of LB Broth with 50 μg/mL kanamycin (ThermoFisher Scientific, Waltham, MA) were inoculated with *E. Coli* expressing the plasmids and were incubated overnight in 250 mL Erlenmeyer flasks. Plasmid was isolated from 4.5 mL of culture per manufacture instructions (E.Z.N.A. Plasmid Mini Kit I; Omega Bio-tek, Norcross, GA). MDCK cells were seeded in 60mm dishes and incubated until reaching 60% confluency. Using the isolated plasmids, MDCK cells were transfected per manufacture instructions (jetPrime DNA transfection kit, Polyplus-transfection SA,

Illkirch, France). Over the course of several weeks, cells were selected using G418 at 800 μg/ml and transgene expression was confirmed via fluorescence through an inverted confocal microscope (TCS SPE-II; Leica Microsystems, Buffalo Grove, IL). The CAHS3 transfection was repeated twice, and the two stably expressing populations named "type M" and "type E". One stably expressing control population was established and will be referred to as AcGFP.

2.3 Desiccation

We seeded 10,000 transfected cells of e type E, type M and AcGFP expressing onto glass coverslips in a 24 well plate with supplemented DMEM. The plate was incubated for 24 h at 37°C. After 24 h, we transferred the plate into the fridge at 4°C for 1 h to slow down the metabolism of the cells. Following cool down, the media was aspirated, and the coverslips were placed into a rack for drying in an enclosed sterile pipette box and then put back into the refrigerator at 4°C for 24 h and 48 h. Following desiccation of their respective times, the coverslips were placed into a new 24 well plate and incubated on ice for 1 h. Following incubation on ice, the plate was transferred into the incubator at 37°C and watched for several days without replacing media. Cell viability was tested with a live/dead cell assay (Biotium, Fremont, CA). Experiment was done in triplicate.

2.4 Epithelial to Mesenchymal Transition Assay

We seeded 10,000 transfected cells of type E, type M and AcGFP expressing onto glass coverslips in their respective pair of wells for the control and experimental well in a 24 well plate with supplemented DMEM. The plate was incubated for 24 h at 37 °C. After

24 h, we aspirated the media off and replaced the media with fresh media or with media supplemented with 15 μM of KY02111 (Cayman Chemical Company, Ann Arbor, MI) respectively. The plate was put back into the incubator for 48 h. Following incubation, the coverslips were fixed in 4% paraformaldehyde and 0.5% Triton-X100 and stained for fibronectin using mouse anti-fibronectin (sc-18825, Santa Cruz Biotechnology, Santa Cruz, CA) and goat anti-mouse conjugated to Dylight 554 (ThermoFisher Scientific). Actin was counterstained using phalloidin conjugated to Dylight 488 (ThermoFisher Scientific) and the nucleus using DRAQ7 (Cell Signaling Technology, Danvers, MA). Following staining, the coverslips were mounted onto glass slides and visualized using confocal microscopy. We imaged an area of ~500 mm^2 and then quantified using custom code (MATLAB 2018b; Mathworks, Natick, MA) that quantified the mean relative expression of fibronectin within identified cellular boundaries. The experiment was done in pentaplicate.

2.5 Thermal Stress

For the thermal stress assay the cell populations were synchronized using a double thymidine block.[13] AcGFP1 and CAHS3 expressing cells were seeded at a density 5,000 cells/well onto glass coverslips in a 24 well plate with supplemented DMEM and incubated overnight. We added thymidine to 2 mM and incubated for 16 h. After 16 h, the media was aspirated off and washed 3x with PBS with Ca+ and Mg+. Following the PBS wash, we added fresh media and incubated for 9 h. Following the 9 h incubation, we repeated the thymidine treatment for another 16 h. The plate was placed at 44.5°C for 1hr at 0 RPM. For controls, coverslips were transferred to another 24 well plate and placed back in the

incubator. Following the heat treatment, the plates were allowed to recover in normal conditions for 48 h. Following incubation, the coverslips were fixed in 4% paraformaldehyde and 0.5% Triton-X100 and stained for fibronectin using rabbit anti-YAP (D8H1X; Cell Signaling Technology) and goat anti-rabbit conjugated to Dylight 554 (ThermoFisher Scientific). Actin was counterstained using phalloidin conjugated to Dylight 488 (ThermoFisher Scientific) and the nucleus using DRAQ7 (Cell Signaling Technology). Following staining, the coverslips were mounted onto glass slides and visualized using confocal microscopy. We imaged an area of ~900 mm^2 and then quantified using custom code (MATLAB 2018b; Mathworks, Natick, MA) that quantified the mean relative expression of YAP within the nucleus and the relative cell area. Heat shock treatments were normalized to that of the control. The experiment was done in quadruplicate.

2.6 Solvent Stress

AcGFP1 and CAHS3 expressing cells were seeded at a density 15,000 cells/well onto glass coverslips in their respective wells in a 24 well plate with supplemented DMEM and incubated overnight. The medium was replaced with freshly prepared media at varying percentages of ethanol or isopropanol: 0,1,5,7 and 10% and incubated for 24 h. Cell viability was assayed for the number of live cells using Calcein-AM per manufacturer instructions (Live/Dead Animal Cell Kit; Biotium, Fremont, CA). Propidium Iodide was similarly used to label dead cells, but was considered unreliable due to the dying cells detaching from the coverslips during the experiment and processing. Cell number and viability were visualized via fluorescence microscopy of a ~500 mm^2 area of each

coverslip. The images were then quantified through custom code on MATLAB to count the cells. The total number of cells found in each image was normalized to that of the mean of the cells at 0% ethanol or isopropanol for AcGFP and CAHS3 respectively. Ethanol study was conducted in quintuplicate while the isopropanol study was conducted in triplicate.

3.0 Results

3.1 Desiccation

In our attempts to subject MDCK cells to similar desiccation conditions to that of the seminal Horiwaka paper in tardigrade's robust survival capabilities, our team managed to successfully desiccate and revive transfected MDCK cells after 48 h, with viable cells proliferating in the culture after approximately 1 week.[1] However, the cells demonstrated a mesenchymal phenotype atypical with normal MDCK cells. These cells exhibited behavior consistent with mesenchymal cells including increased migration, loss of cell to cell adhesion and elongated processes (**Fig. 1**) in contrast to the more epithelial phenotype of AcGFP-expressing cells.

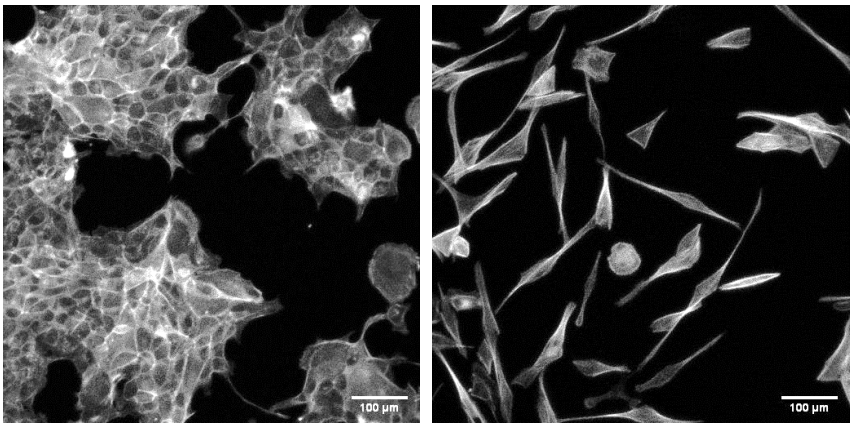

Figure 1. Actin Stain of Transfected Cells *(A)Actin stain of AcGFP and expressing MDCK cells. (B) Actin stain of CAHS3 expressing MDCK cells designated as CAHS3 type M.*

It is important to note that the desiccation experiment could not be replicated despite several attempts. To confirm this was not an invasive cell contamination, we tested

for the presence of canine GAPDH through qPCR and confirmed the presence of canine GAPDH, suggesting that the cells were MDCK as that is the only canine cell in the lab. Furthermore, the transfection was done once more and primarily an epithelial morphology was observed with an occasional mesenchymal morphology. Therefore, we believe that CAHS3 transfected cells may have had two morphologies, one of which survived desiccation for unknown reasons. The new cell morphology, designated type M, was further investigated for stress tolerance and mesenchymal markers. The alternate CAHS3 expressing strain, that did not undergo desiccation and maintained an epithelial phenotype, was designated type E and assayed for stress tolerance.

It is worth noting that type M appeared to exhibit a marginal increased expression of CAHS3 through their intensity when observed under the confocal microscope. For purposes of this thesis, both type E and type M populations are used for a more holistic investigation on the effects of CAHS3 on stress tolerance with an emphasis on type E.

3.2 Epithelial to Mesenchymal Transition Experiment

The following experiment was conducted as an investigation into determining if the Wnt pathway was affected by CAHS3 expression giving rise to mesenchymal morphology. First, when comparing AcGFP expressing MDCK cells to those expressing CAHS3, we noticed that that was a 22% increase in fibronectin among type M cells (**Fig. 2A**).

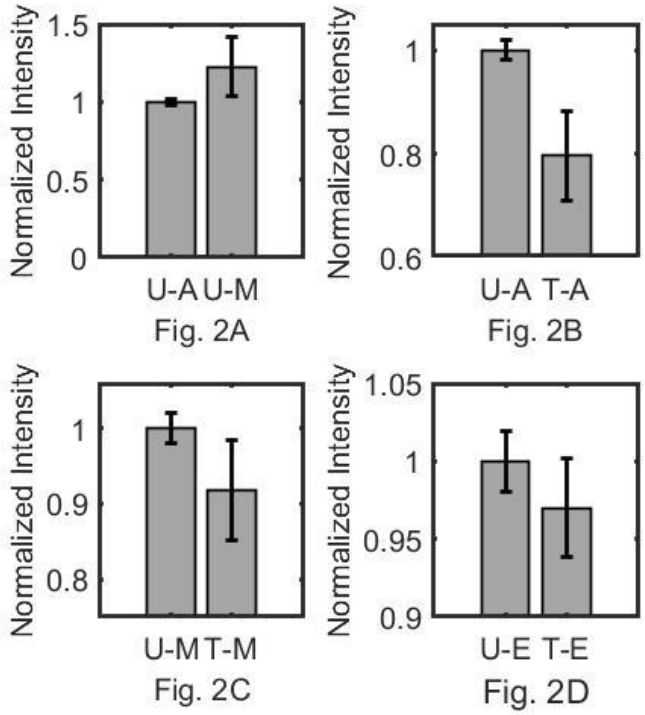

Figure 2. Fibronectin Stain *(A) Comparison of relative normalized fibronectin expression via confocal microscopy between untreated AcGFP [U-A] expressing and untreated type M cells [U-M]. (B)(C)(D) Comparison of normalized fibronectin expression between untreated [U] and KY02111 treated [T] AcGFP[A], type M[M] and type E [E] cells.*

Secondly, we also noticed a further decrease in fibronectin among the KY02111 treated AcGFP expressing cells compared to that of the nontreated population demonstrating the control cell type was behaving as expected.

Lastly, we observed that the administration of 15 μM KY02111 didn't shift the expression of fibronectin among the CAHS3 M type and E type population by a margin beyond the deviation of the untreated cells.

A student t-test was conducted on the experiment populations to their controls and transcribed in Table 1 with a desired confidence level set to 95%.

Table 1. EMT Fibronectin Comparison Student T-test Probability of Dissimilarity Results			
Untreated AcGFP vs Treated AcGFP	*AcGFP vs M type*	*Untreated M Type vs Treated M Type*	*Untreated E Type vs Treated E Type*
0.901689	0.68088	0.690268	0.587458

3.3 Thermal Stress

The expression of CAHS3 appears to mitigate the effects of heat shock related senescence by a reducing the overall increase in cell area/cell by nearly 40.6% versus that of the AcGFP population (**Fig. 3A**). However, we did not notice any appreciable difference in nuclear YAP localization (**Fig. 3B**). We did, however, also notice a 10% mitigation of decline in cell population between AcGFP expressing cells and the E type (**Fig. 4)**.

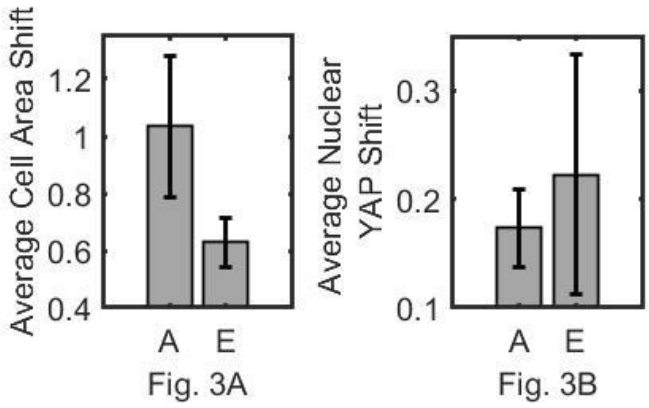

Figure 3. Heat Shock *(A) Average percentage shift of nuclear YAP/ pixel of AcGFP and CAHS3 Type E expressing cells undergoing heatshock from that of non-heat treated cells. (B) Average percentage shift of cell area of AcGFP and CAHS3 Type E expressing cells undergoing heatshock from that of non-heat treated cells.*

A student t-test was conducted on the experiment populations to their controls and transcribed in Table 2 with desired confidence level set to 95%.

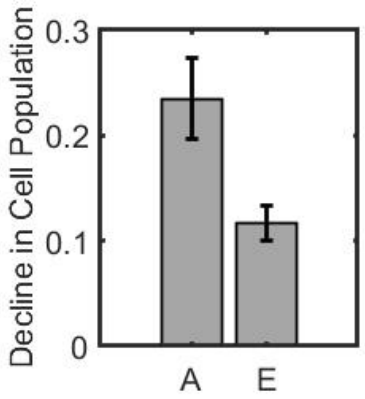

Figure 4. Heat Shock *Percentage decline in cell population in AcGFP cells versus E type cells as a result of heat shock*

Table 2. Heat Shock Student T-test Probability of Dissimilarity Results		
Cell Population	*Cell Area*	*YAP*
0.974858	0.575205	0.359463

3.4 Solvent Stress

After visualizing through the confocal, ethanol and isopropanol treatments at various percentages yielded a curious result. When comparing AcGFP expressing MDCK cells to those expressing CAHS3 of both type E and type M

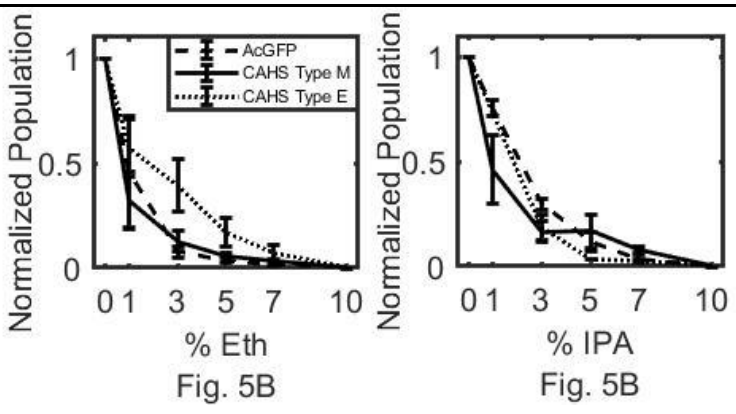

Figure 5. Cell Viability under Solvent Exposure. *Percentage of living cells in proportion to concentration of alcohol.*

we noticed that that was a sharp decline in cell viability among all populations at 1% ethanol however, type E diverged and retained greater viability from 39.3% to 17.1% under 3% to 5% ethanol respectively (**Fig. 4A**). However, at higher concentrations of ethanol past 7%, all cell populations possessed similar viability below 1%. Isopropanol treatments did not result in any population faring better than the other. Type E and type M either performed similarly or worse than that of the AcGFP expressing population.

A student t-test was conducted on the experiment populations to their controls and transcribed in Table 3 and 4 for ethanol and isopropanol respectively with amdesired confidence level set to 95%.

Table 3. Ethanol Population Decline Student T-test Probability of Dissimilarity Results						
	0% Eth	*1% Eth*	*3% Eth*	*5% Eth*	*7% Eth*	*10% Eth*
AcGFP vs M Type	0	0.331198	0.80782	0.836034	0.349257	0
AcGFP vs E Type	0	0.447461	0.91939	0.860287	0.743121	0

Table 4. Isopropanol Population Decline Student T-test Probability of Dissimilarity Results						
	0% IPA	*1% IPA*	*3% IPA*	*5% IPA*	*7% IPA*	*10% IPA*
AcGFP vs M Type	0	0.756009	0.789105	0.587992	0.428069	0
AcGFP vs E Type	0	0.995943	0.989856	0.786433	0.901076	0

4.0 Discussion

4.1 Desiccation

We analyzed the efficacy of TDPs to increase stress tolerance by the measure of viability and protein expression after desiccation, exposure to organic solvents, and heat

shock. Unfortunately, we could not measure the efficacy of CAHS3 expression on increasing tolerance against desiccation due to type M CAHS3 expressing cells being the only recovered cells and only in a singular instance. Any instance to replicate ended in complete loss of cell viability, and it remains unclear if this was an extremely rare event or experimental artifact. Furthermore, this led us to believe that since the mesenchymal morphology might be much rarer, that any prior transfected cell might have been mistaken for an unhealthy or atypical MDCK cells and therefore undocumented in literature.

However, even if CAHS proteins are necessary for protection against desiccation stress, it is reasonable to conclude they alone are not sufficient. As mentioned earlier, there are multiple classes of TDPs that are important in the tardigrade's ability to withstand desiccation. When either SAHS or CAHS is targeted via RNAi and desiccated, their survival drops dramatically by up to 90%.[7] Furthermore, the TDP mitochondrial abundant heat soluble (MAHS) protein has been also found to upregulate during desiccation and to improve osmotic tolerance in other cell types.[7,22] Future work will test the efficacy of expressing multiple classes of TDP in providing desiccation tolerance in mammalian cells. However, since the cells successfully recovered were confirmed to be of canine origin and undoubtedly endured significant stress, we moved on to investigate to cause of the change in morphology present in type M CAHS3 expressers.

4.2 Epithelial to Mesenchymal Transition Experiment

We noticed that the type M that had survived desiccation in the singular instance mentioned before had a mesenchymal morphology (for which they were aptly designated

as type M), which qualitatively is signified by the loss of tight junction with adjacent cells, migratory behavior and an enlarged, elongated cell morphology (**Fig. 1B**).[24,25] To understand this change in morphology, we investigated the epithelial to mesenchymal transition (EMT) phenomenon. EMT is a phenomenon is found in aggressive cancer strains where epithelial cells lose certain markers and begin expressing markers typically associated with mesenchymal morphologies.[26] Increases of mesenchymal markers, such as fibronectin, also can drive the shift to a mesenchymal morphology.[26–28] To determine if CAHS3 expression in type M was associated with typical EMT, we inhibited the Wnt pathway using the pharmacological inhibitor KY02111. Activation of the Wnt pathway is essential for EMT furthermore, KY02111 is known to inhibit the Wnt pathway.[29–31] To investigate the cause of change in morphology, we administered KY02111 treatment to shift the expression of fibronectin, a mesenchymal marker. We first confirmed that there was a greater relative expression of fibronectin in untreated AcGFP versus KY02111 treated AcGFP cells with a statistically significant difference in population by 97.5% which demonstrates successful controls (**Table 1**). Next we compared CAHS3 type M populations compared to that of the AcGFP populations and found a 20% increase in relative fibronectin expression that found to be statistically insignificant with only 68.1% confidence (**Table 1)**. More replicates would be required to gain determine similarity. We then observed that KY02111 treatment was unable able to alter fibronectin expression in both the type E and type M MDCK cells compared to the untreated populations (**Fig. 2C & Fig. 2D**). We hypothesize either the Wnt pathway isn't involved in CAHS3-induced EMT or that the Wnt pathway is so strongly upregulated by CAHS3 that KY02111 has

minimal effect. Other pathways involved in EMT that could be explored in future work include Notch, Hedgehog, transforming growth factor β, and tyrosine kinases.[32–34] We then moved onward to main purpose of this study: to investigate CAHS3's effect on increasing stress tolerance.

4.3 Thermal Stress

CAHS3 proteins belong to a larger class of LEA-like TDP proteins that help stabilize the folding of proteins and furthermore prevent the aggregation of misfolded proteins during stress such as heat shock.[2,5,6,8] Misfolded protein aggregation leads to cell death due to interference with critical pathways.[12] While there was no difference in relative YAP expression as a result of heat shock between type E and AcGFP expressing cells, there was a 40.6 % difference in decreased cell size and 10% increase in cell death difference in type E versus AcGFP expressing cells (**Fig. 3 and Fig 4**). The decrease in cell size as a result of heat shock in type E cells was statistically insignificant with only 57.5% confidence meanwhile, the increase in cell death among type E cells during heat shock was statistically significant with 97.5% confidence. As increased cell size is a hallmark of senescence, it is possible CAHS3 mitigates the entry into senescence but more replicates are required to elucidate any trends.[18] Although the similar levels of YAP appears to lead to an opposing conclusions compared to the cell area findings, it should be clarified that the cells *remaining* have similar relative levels of YAP. We must keep in mind that CAHS3 might prevent the aggregation of proteins, similar to role HSP90, and evidence in literature suggest that decreases in HSP90 lead to increased YAP nuclear

localization. The function of nuclear localization of YAP is to prevent cells from going down an apoptotic pathway rather than remain in a senescent state, and so then we can deduce that CAHS3 might have prevented YAP localization causing apoptosis and resulting in a greater cell death among the type E population rather than that of the AcGFP population.[7,19,23] The reduced rate of increased cell size, however, is still very promising to suggest the protective capabilities of CAHS3 in mammalian cells.

4.4 Solvent Stress

In the same manner of protein stabilization in the context of thermal stress, we sought to investigate CAHS3's effects on mitigating the toxic effects of organic solvents such as ethanol and isopropanol. Alcohols and other organic solvents are well understood to be quite toxic to mammalian cells, with doses of 10% leading to complete cell loss.[35] As tardigrades were demonstrated to withstand up to 90% acetonitrile in the Horikawa paper, we tested the viability of CAHS3 expressing MDCK cells in the presence of ethanol for 24 h.[1] Alcohols are known to disorder the lipids of the cellular membrane and increase the membrane/buffer partition coefficient with longer chain lengths of alcohol associated with increased damage.[36] Furthermore, ethanol modifies the structure of proteins by modifying the β-sheet and/or β-turn structure and also inhibits protein formation during the elongation phase of RNA sythesis by direct inhibition of a sythentase.[37,38] Our data show that type E CAHS3 expressing cells show increased tolerance to ethanol at 3% and 5% ethanol but not isopropanol. The increased survival at 3% and 5% ethanol were found to be not statistically significant reaching 80.8% and 91.9% confidence respectively . This is somewhat

promising when accounting for the number of samples and more replicates should be conducted. We hypothesized that the increase in survival is due to the increased toxicity of longer alcohol chains that might cause membrane and protein instability beyond that which the molecular chaperone function of CAHS3 can handle; more solvents should be investigated.[6–9,22] The stabilization that CAHS3 confers might be quintessential to increasing stress tolerance during exposure to organic solvents such as ethanol.

4.6 Limitations

A key limitation of our findings was the set up for the desiccation experiment. We were unable to control the pressure or rate of desiccation in a consistent manner due to equipment available. Furthermore, due to time constraints we were unable to express other tardigrade proteins within the cell that might have potentially improved its rate of survival. Literature has shown that the gene silencing of other critical TDP proteins such as MAHS and SAHS and their analogs show a sharp decrease in desiccation survival.[7] These TDP proteins may interact synergistically inside the tardigrade to allow it to survive desiccation. Perhaps the expression of the various TDP proteins might help in the desiccation survival of mammalian cells. Furthermore, in regard to the heat shock experiments, the measurement of misfolded proteins, HSPs, and LATS1 should be investigated to more precisely determine CAHS3's stabilizing affects.

4.7 Conclusion

We have demonstrated that the expression of CAHS3 improves the stress tolerance of MDCK cells and quite potentially other mammalian cells types. We have shown that

CAHS3 may stabilize mammalian cells from ethanol and may mitigate the effects of heat-shock induced senescence. This work suggests that TDP expression can be a tool for creating robust cell lines for projects or experiments that would otherwise cause great stress to the cells. These robust cell lines may improve biomanufacturing of toxic products and increase lab's capabilities of creating durable tissue constructs.

References

1. Horikawa, D. D. *et al.* Establishment of a Rearing System of the Extremotolerant Tardigrade *Ramazzottius varieornatus* : A New Model Animal for Astrobiology. *Astrobiology* **8**, 549–556 (2008).
2. Tapia, H. & Koshland, D. E. Trehalose is a versatile and long-lived chaperone for desiccation tolerance. *Curr. Biol.* **24**, 2758–2766 (2014).
3. Jain, N. K. & Roy, I. Effect of trehalose on protein structure. *Protein Sci* **18**, 24–36 (2009).
4. Ruhal, R., Kataria, R. & Choudhury, B. Trends in bacterial trehalose metabolism and significant nodes of metabolic pathway in the direction of trehalose accumulation. *Microb Biotechnol* **6**, 493–502 (2013).
5. Hengherr, S., Heyer, A. G., Köhler, H.-R. & Schill, R. O. Trehalose and anhydrobiosis in tardigrades – evidence for divergence in responses to dehydration. *The FEBS Journal* **275**, 281–288 (2008).
6. Chakrabortee, S. *et al.* Hydrophilic protein associated with desiccation tolerance exhibits broad protein stabilization function. *PNAS* **104**, 18073–18078 (2007).
7. Boothby, T. C. *et al.* Tardigrades Use Intrinsically Disordered Proteins to Survive Desiccation. *Molecular Cell* **65**, 975-984.e5 (2017).
8. Yamaguchi, A. *et al.* Two Novel Heat-Soluble Protein Families Abundantly Expressed in an Anhydrobiotic Tardigrade. *PLOS ONE* **7**, e44209 (2012).
9. Piszkiewicz, S. *et al.* Protecting activity of desiccated enzymes. *Protein Science* **28**, 941–951 (2019).
10. Weids, A. J., Ibstedt, S., Tamás, M. J. & Grant, C. M. Distinct stress conditions result in aggregation of proteins with similar properties. *Scientific Reports* **6**, 24554 (2016).

11. Benzeno, S. & Diehl, J. A. A novel WISp39 protein links Hsp90 and p21 stability to the G $_2$ /M checkpoint. *Cancer Biology & Therapy* **4**, 382–384 (2005).

12. Jascur, T. *et al.* Regulation of p21WAF1/CIP1 Stability by WISp39, a Hsp90 Binding TPR Protein. *Molecular Cell* **17**, 237–249 (2005).

13. Mechanism of heat stress-induced cellular senescence elucidates the exclusive vulnerability of early S-phase cells to mild genotoxic stress. Available at: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4513856/. (Accessed: 18th August 2019)

14. Alford, B. D. & Brandman, O. Quantification of Hsp90 availability reveals differential coupling to the heat shock response. *J Cell Biol* **217**, 3809–3816 (2018).

15. Li, Z. & Srivastava, P. Heat-shock proteins. *Curr Protoc Immunol* **Appendix 1**, Appendix 1T (2004).

16. Vabulas, R. M., Raychaudhuri, S., Hayer-Hartl, M. & Hartl, F. U. Protein Folding in the Cytoplasm and the Heat Shock Response. *Cold Spring Harb Perspect Biol* **2**, (2010).

17. Yang, J. *et al.* Cell size and growth rate are major determinants of replicative lifespan. *Cell Cycle* **10**, 144–155 (2011).

18. Excessive Cell Size Contributes to Senescence | | LEAF.

19. Ma, K. *et al.* Nuclear accumulation of Yes-Associated Protein (YAP) maintains the survival of doxorubicin-induced senescent cells by promoting survivin expression. *Cancer Lett.* **375**, 84–91 (2016).

20. Heat Shock Protein 90 Inhibition Depletes LATS1 and LATS2, Two Regulators of the Mammalian Hippo Tumor Suppressor Pathway | Cancer Research. Available at: https://cancerres.aacrjournals.org/content/70/21/8642.long. (Accessed: 29th August 2019)

21. YAP Nuclear Localization in the Absence of Cell-Cell Contact Is Mediated by a Filamentous Actin-dependent, Myosin II- and Phospho-YAP-independent Pathway during

Extracellular Matrix Mechanosensing. Available at: http://www.jbc.org/content/291/12/6096.full. (Accessed: 29th August 2019)

22. Tanaka, S. *et al.* Novel Mitochondria-Targeted Heat-Soluble Proteins Identified in the Anhydrobiotic Tardigrade Improve Osmotic Tolerance of Human Cells. *PLoS One* **10**, (2015).

23. Goyal, K., Walton, L. J. & Tunnacliffe, A. LEA proteins prevent protein aggregation due to water stress. *Biochemical Journal* **388**, 151–157 (2005).

24. Martin, F. T. *et al.* Potential role of mesenchymal stem cells (MSCs) in the breast tumour microenvironment: stimulation of epithelial to mesenchymal transition (EMT). *Breast Cancer Res Treat* **124**, 317–326 (2010).

25. Kasai, H., Allen, J. T., Mason, R. M., Kamimura, T. & Zhang, Z. TGF-β1 induces human alveolar epithelial to mesenchymal cell transition (EMT). *Respiratory Research* **6**, 56 (2005).

26. Li, C.-L. *et al.* Fibronectin induces epithelial-mesenchymal transition in human breast cancer MCF-7 cells via activation of calpain. *Oncol Lett* **13**, 3889–3895 (2017).

27. Wang, J. P. & Hielscher, A. Fibronectin: How Its Aberrant Expression in Tumors May Improve Therapeutic Targeting. *J. Cancer* **8**, 674–682 (2017).

28. Zeisberg, M. & Neilson, E. G. Biomarkers for epithelial-mesenchymal transitions. *J Clin Invest* **119**, 1429–1437 (2009).

29. Tanabe, S. Wnt Signaling and Epithelial-Mesenchymal Transition Network in Cancer. 2

30. Schade, D. & Plowright, A. T. Medicinal Chemistry Approaches to Heart Regeneration. *J. Med. Chem.* **58**, 9451–9479 (2015).

31. Liu, K., Yu, C., Xie, M., Li, K. & Ding, S. Chemical Modulation of Cell Fate in Stem Cell Therapeutics and Regenerative Medicine. *Cell Chemical Biology* **23**, 893–916 (2016).

32. Zhou, P. *et al.* The epithelial to mesenchymal transition (EMT) and cancer stem cells: implication for treatment resistance in pancreatic cancer. *Molecular Cancer* **16**, 52 (2017).

33. Liu, X. *et al.* Roles of Signaling Pathways in the Epithelial-Mesenchymal Transition in Cancer. *Asian Pac. J. Cancer Prev.* **16**, 6201–6206 (2015).

34. Gonzalez, D. M. & Medici, D. Signaling mechanisms of the epithelial-mesenchymal transition. *Sci Signal* **7**, re8 (2014).

35. Tapani, E., Taavitsainen, M., Lindros, K., Vehmas, T. & Lehtonen, E. Toxicity of Ethanol in Low Concentrations: Experimental evaluation in cell culture. *Acta Radiol* **37**, 923–926 (1996).

36. Goldstein, D. B. Effect of alcohol on cellular membranes. *Ann Emerg Med* **15**, 1013–1018 (1986).

37. Lin, S.-Y., Wu, C.-W. & Liang, R.-C. Effect of Ethanol on the Protein Secondary Structure of the Human Gastric Mucosa, In Vitro. *Clinical Chemistry and Laboratory Medicine* **33**, (1995).

38. David, E. T. & Fischer, I. Studies on theEffect of Ethanol on EukaryoticProtein Synthesis in Vitro. 6