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Trehalose Transporter from African Chironomid Larvae Improves Desiccation Tolerance of Chinese Hamster Ovary Cells

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Abstract:

Dry preservation has been explored as an energy-efficient alternative to cryopreservation, but the high sensitivity of mammalian cells to desiccation stress has been one of the major hurdles in storing cells in the desiccated state. An important strategy to reduce desiccation sensitivity involves use of the disaccharide trehalose. Trehalose is known to improve desiccation tolerance in mammalian cells when present on both sides of the cell membrane. Because trehalose is membrane impermeant the development of desiccation strategies involving this promising sugar is hindered. We explored the potential of using a high-capacity trehalose transporter (TRET1) from the African chironomid *P. vanderplanki* (Kikawada et al. 2007) to introduce trehalose into the cytoplasm of mammalian cells and thereby increase desiccation tolerance. When Chinese Hamster Ovary cells (CHO) were stably transfected with TRET1 (CHO-TRET1 cells) and incubated with 0.4 M trehalose for 4 h at 37 ^{0}C, a seven-fold increase in trehalose uptake was observed compared to the wild-type CHO cells. Following trehalose loading, desiccation tolerance was investigated by evaporative drying of cells at 14 % relative humidity. After desiccation to 2.60 g of water per gram dry weight, a 170 % increase in viability and a 400 % increase in growth (after 7 days) was observed for CHO-TRET1 relative to control CHO cells. Our results demonstrate the beneficial effect of intracellular trehalose for imparting tolerance to partial desiccation.

Introduction:

Current techniques for long-term preservation of mammalian cells depend on cryogenic temperatures and have many disadvantages including complicated procedures, high cost of maintenance and limited transportation ability (Acker et al. 2004, Brockbank and Taylor, 2007). Preservation of biological materials in the dry state at ambient temperature is an alternative approach to address the growing demand for cell replacement technologies and regenerative medicine. However, mammalian cells are highly sensitive to desiccation stresses and the viability of cells decrease rapidly upon drying. Recent research has focused on learning from the adaptive strategies displayed by species that are naturally desiccation tolerant in an attempt to biomimic these in mammalian cells (Ma et al., 2005, Menze et al, 2010)

Many anhydrobiotic organisms in nature are capable of withstanding extreme desiccation. Examples of such organisms include plant seeds, bacteria, yeast, nematodes, and cysts of certain crustaceans (Yancey et al., 1982; Crowe et al., 1992). Some of these organisms can survive the removal of more than 99 % of their body water (Crowe et al., 1998). Trehalose is found to accumulate in high concentrations (up to 20% of the dry weight) in many of these anhydrobiotic organisms during desiccation (Crowe et al. 2000), and it is believed to play a major role in imparting desiccation tolerance to anhydrobiotic organisms at the cellular level (Crowe et al. 1987, Crowe et al., 1998). However, as trehalose is impermeant to mammalian cells, a variety of techniques have been explored in order to find an efficient mechanism to introduce sugars, like trehalose, into the interior of mammalian cells. These include transfection (Guo et al., 2000), engineered pores (Eroglu et al., 2000; Russo et al., 1997), activation of native channels (Elliott et al., 2006), microinjection (Eroglu et al., 2003), and endocytosis (Hubel and Darr 2002; Oliver et al., 2004). Recent studies showed that anhydrobiotic insect larvae utilize

specific trehalose transporters during desiccation stresses. The trehalose transporter (TRET1) from the anhydrobiotic larvae of African chironomid, *Polypedilum vanderplanki* has been isolated and characterized by Kikawada and his coworkers (Kikawada et al. 2007; Kanamori et al. 2010).

In this study, the TRET1 was stably expressed in CHO cells (CHO-TRET1) and then trehalose introduced into the cells by simple incubation in a trehalose containing medium. Incubation conditions were optimized for exposure time and trehalose concentration, and intracellular trehalose was quantified using high performance liquid chromatography (HPLC). After partial desiccation, significant increases in viability and growth were observed for CHO-TRET1 cells relative to control CHO cells.

Materials and Methods

Cell Culture

Chinese hamster ovary cells (CHO) were obtained from the American Type Culture Collection (Manassas, VA) and cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco Life Trechnologies, Gaithersburg, MD) supplemented with 10% fetal bovine serum (Atlanta Biologicals, Norcross, GA) and 2% penicillin-streptomycin (10 U/mL penicillin G and 10 ug/mL streptomycin sulfate, Gibco). Cultures were maintained at 37°C, and equilibrated with 10% CO_2-90% air in 25-cm^2 cell culture T-flasks (Corning Incorporated, NY).

Transfection of Trehalose Transporters

The trehalose transporter (TRET1) expression vector (pPvTRET1-IRES2-AcGFP1) for mammalian cell expression was kindly provided by Dr. Takahiro Kikawada from National Institute of Agrobiological Sciences at Ibaraki, Japan. The TRET1 sequence was fused with a green fluorescent protein (GFP) tag at the C-terminus by subcloning into the vector pIRES2-AcGFP1 (Clonetech, Mountain View, CA) for the generation of a stable cell line (pPvTRET1-IRES2-AcGFP1). Wild-type CHO cells were transfected with pPvTRET1-IRES2-AcGFP1 using Lipofectamin 2000 and stably transfected cells were selected with Geneticin (Invitrogen, Carlsbad, CA) until colonies of drug resistant cells appeared in the culture dishes.

Optimization of Trehalose Loading

When cells reached about 80% confluence, the cell culture medium in the tissue culture flask was completely removed using a Pasteur pipette with vacuum aspirator and immediately replaced with culture media that was supplemented with 100, 250 and 400 mM trehalose. Cells were then incubated for 1, 2 and 4 h under the incubation conditions describe above. Cells were

then detached from the tissue culture flasks by trypsinization (0.25% trypsin, 1 mM EDTA solution) and collected by centrifugation at 100 g for 10 min. The cell samples were washed three times in phosphate buffered saline (PBS), and then lysed by freeze-thawing in 18 mOhm high-purity water. The solution containing lysed cells was centrifuged, and the supernatant was collected and filtered for high performance liquid chromatography analysis. Analyses were performed using a Dionex HPLC system with a GP-50 gradient pump, an EC50 electrochemical detector and a PA10 column (Dionex, Sunnyvale, CA). An AS50 auto-sampler and thermal compartment was used for sample handling and injection during analysis. Sample peaks were identified by comparison of retention times to standards, and calibration curves were linear over the range assayed ($r^2 = 0.92$). Kinetic analysis of trehalose uptake into the intracellular space was performed at multiple external trehalose concentrations to determine the affinity of TRET1. Data were analyzed using Sigma Plot 11.1 Enzyme Kinetics Module (Systat Software Inc., IL).

Drying Protocol

Samples were prepared for dry processing by plating CHO and CHO-TRET1 cells onto 22-mm glass coverslips (Fisher Scientific, Pittsburgh, PA) in 20 µl droplets, at a density of 5×10^5 cells/ml (10,000 cells per droplet). The coverslips were then placed in 35 mm tissue culture dishes (BD Biosciences, Research Triangle Park, NC) and returned to the incubator for 45 min to allow cell attachment. Following the incubation period, the media was gently aspirated and an equal volume of drying buffer (200 mM trehalose, 120 mM NaCl, 10mM KCl, 5 mM glucose, 20 mM HEPES, pH 7.4) was placed on top of the plated cells. These coverslips were then weighed and placed in a Secador autodesiccator (Fisher Scientific, Pittsburgh, PA) for various periods ranging from 10 to 120 min. A wide range of drying times was used to ensure that a fairly large range of water contents could be measured over the course of a single experiment.

The moisture content of the samples were determined gravimetrically, and expressed as a ratio of remaining water mass in the sample to the anhydrous dry weight (g H_2O/g dry weight). The anhydrous dry weight was measured after baking 5 representative samples at 110 ^{0}C for at least 24 h.

Cell Rehydration and Membrane Integrity Assessment

Following desiccation, samples were rehydrated using 100 µl of cell culture medium and allowed to recover for 20 minutes at 37 °C and 5% CO_2. After the recovery period the supernatant was carefully removed (leaving behind the cells which were attached to the surface of the cover slip) and the membrane integrity of detached cells was determined by staining with 0.4 % Trypan Blue solution (Fisher Scientific, Pittsburgh PA). The membrane integrity of the attached cells was determined using a SYTO 13 and propidium iodine membrane-integrity assay (Molecular Probes, Eugene, OR). The stock solution was prepared by adding 10 µl of a 1 mg/ml SYTO 13 solution (aq.) and 5 µl of a 1.0 mg/ml propidium iodine solution (aq.) to 10 ml of phenol-red and FBS free DMEM (Invitrogen Corporation, Carlsbad, CA). An aliquot of 130 µl of this solution was then added to the attached cells on the coverslip and imaged using an inverted Zeiss microscope (Thornwood, NY) equipped with dual fluorescence filter set (51004-V2, Chroma Technology, Brattleboro, VT). The membrane integrity of the attached cells was determined by counting the live (green) and dead (red) cells on three representative images from the same sample. The total membrane integrity was estimated by taking in account both detached cells and attached cells (for further details, cf. Chakraborty et al. 2008).

Long-term Growth Studies

Following rehydration, the cells were transferred to a 60 mm cell culture dish (Corning Incorporated, Corning, NY) and cultured for 7 days. Cell counts were performed in parallel samples on 1^{st}, 3^{rd} and 7^{th} day of incubation following rehydration ($n = 5$). Cells were collected by trypsinization and were counted in a hemocytometer (Fisher Scientific, Pittsburgh PA), using 0.4 % Trypan Blue (Sigma–Aldrich, St. Louis, MO) exclusion as an indicator of membrane integrity and viability.

Colony Forming Unit Assays

Proliferation of desiccated cells was studied by measuring clonogenic output by counting colony-forming units (CFU). Desiccated cell samples were rehydrated using fully complemented medium at 37 ^{0}C. After a 24 h recovery period on glass slides, the cells were trypsinized, counted, and approximately 1000 cells/mL were transferred to cell 60 mm culture plates for colony studies. All samples were cultured at 37°C in humidified air containing 10% CO_2 for 7 days, after which colonies were fixed using 3.7% paraformaldehyde (PFA) solution and stained using 0.05% crystal violet in distilled water for 30 min. The cell culture dishes were then photographed and colonies enumerated using an inverted Zeiss microscope (1 colony contains a minimum of 50 cells).

Results

CHO cells stably expressing a GFP-labeled trehalose transporter from the African chironomid *P. vanderplanki* are morphological indistinguishable from wild type CHO cells (Fig. 1A). The micrographs were taken at the same stage of confluency and the GFP tagged transporters are visible in the fluorescence micrograph (wavelength 490 nm) while no such transporters are seen in wild type CHO cells (Fig. 1A). HPLC analysis was performed on cell extracts after incubation for different time periods with various extracellular trehalose concentrations. Incubation of the CHO-TRET1 cells in 400 mM trehalose solution for 4 h resulted in an uptake of 23.45 ± 2.67 mM trehalose into the cells (Fig. 2A). The intracellular trehalose concentration was estimated using the average volume of the CHO cells (696.56 μm^3) and considering 70% of the cell volume to be osmotically active. Figure 2B demonstrates a correlation between the concentration of intracellular trehalose and the viability of the cells. As the data indicate, the increased extracellular osmolality of 400 mM trehalose in the cell culture medium was well tolerated by the cells, and cell viability did not decrease appreciably. However, when the cells were exposed to 600 mM trehalose for 6 h, ~40% of the cell population lost their viability (data not shown).

Kinetic analysis was used to characterize trehalose uptake into the intracellular space. Assuming one binding site per transporter, the apparent values for K_m and V_{max} were found to be 137 ± 87 mM and 192 ± 44 pmol/min/10^6 cells, respectively (Fig. 3). The rather high K_m for trehalose indicates low affinity for substrate binding.

After trehalose loading, cells were dried in a low humidity chamber for various times to achieve a wide range of final moisture contents. The fraction of CHO cells that were able to preserve their membrane integrity following rehydration is shown in Figure 4, and expressed as a

function of moisture content (gH_2O/gdw). CHO-TRET1 cells incubated in 0.4 M trehalose solution for 4 h were able to withstand a greater degree of moisture loss than untreated cells (F= 215.45, $p < 0.05$) and CHO-TRET1 cells that were not loaded with trehalose showed moister-dependent decreases in membrane integrity similar to wild-type CHO cells (data not shown). Defining LD_{50} as the degree of dryness that causes half of the initial cell population to lose membrane integrity (indicator of lethality), CHO-TRET1 cells pre-treated with trehalose had a LD_{50} of ~1.9 gH_2O/gdw, whereas cells that were not pre-treated with trehalose had a LD_{50} of ~4 gH_2O/gdw. Thus CHO-TRET1 cells pre-treated with trehalose can tolerate twice the level of dryness than cells that were not loaded with the sugar. The membrane integrity of the trehalose-loaded CHO-TRET1 cells gradually decreased until the moisture content of the sample reached 2 gH_2O/gdw. Below this moisture level, the membrane integrity of the cells decreased rapidly. CHO cells that were not pre-treated with trehalose and dried to moisture contents of 4.32 ± 0.73 and 3.5 ± 0.17 gH_2O/gdw, showed membrane integrities of 38.66 ± 2.75 and 22.9 ± 2.8 % (mean ± SE, n = 5), respectively. CHO and CHO-TRET1 cells pre-treated with 400 mM trehalose for 4 h and dried to 4.32 ± 0.73 gH_2O/gdw showed membrane integrities of 57.8 ± 8.7 % and 76.9 ± 8.3 %, respectively.

The long-term response of cells incubated in 0.4 M trehalose solution for 4 h and desiccated to different moisture contents was analyzed as the number of viable cells in the rehydrated samples as a function of day's post-rehydration (Fig. 5). Viability was quantified for four distinct cell groups with increasing dryness (8.12 ± 0.52, 6.16 ± 0.33, 4.32 ± 0.73 and 2.60 ± 0.52 gH_2O/gdw). Cell viability was quantified on days 1, 3 and 7. The fractional increase in cell number was compared to non-desiccated control cells. CHO-TRET1 cells outperformed wild type CHO cells under all conditions investigated. It is interesting to note that in most cases, the

difference in growth pattern between CHO-TRET1 cells and the CHO cells become prominent by day 3 (72 h). After 7 days of growth under normal culture conditions, the CHO-TRET1 samples desiccated to ~2.60 gH_2O/gdw exhibited almost a four-fold increase in number of cells compared to CHO samples. However, at higher moisture contents of 4.32, 6.16 and 8.12 gH_2O/gdw the relative fractional increases in number of cells were lower with 2.5, 1.5 and 1.6, respectively. Colony forming ability was assessed for previously desiccated samples of CHO-TRET1 and CHO cells to investigate their ability to proliferate after rehydration. Colony counts show a significant difference in number of CFUs between CHO-TRET1 and CHO cells desiccated to 4.32 and 6.16 gH_2O/gdw (Fig. 6). While CHO-TRET1 cells having intracellular trehalose demonstrated some colony forming ability (CFU count 1.3 ± 1.5, n = 3) when desiccated to moisture contents of 2.60 gH_2O/gdw, CHO cells under similar conditions (incubated in trehalose solution prior to desiccation) failed to demonstrate any colony growth (data not shown). There was no significant difference in CFU counts between CHO and CHO-TRET1 control cells (never desiccated) cultured under normal conditions (data not shown). In summary, these data indicate that CHO-TRET1 cells containing intracellular trehalose were able to withstand desiccation stress better compared to control CHO cells.

Discussion

Transgenic expression of TRET1 from *P. vanderplanki* has been shown to facilitate transport of trehalose into Xenopus oocytes (Kikawada et al 2007). In the present study, our objective was to study whether desiccation tolerance of mammalian cells can be improved by taking advantage of the trehalose transport ability of TRET1. The loading characteristics of trehalose into the CHO cells expressing TRET1 transporters were determined by incubating the cell samples in various concentrations of trehalose. The uptake data were then fitted to the Michaelis–Menten and Eadie–Hofstee equations to provide insight into the nature of the uptake mechanism (Fig. 3). The data show that trehalose uptake was influenced by (a) length of incubation and (b) concentration of trehalose in the incubation solution. Consistent with the findings of Kikawada and his co-workers (Kikawada et al. 2007), the K_m value for TRET1 in CHO cells was high (137 mM). Since trehalose accumulates in *P. vanderplanki* under desiccation stress to above 100 mM (Watanabe et al. 2002) the K_m value reported here is in good agreement with the naturally occurring concentrations. As previously indicated by Kikawada et al. (2007), TRET1 has a low substrate affinity compared to other known sugar transporters in mammalian cells such as GLUT1 (~3 mM glucose), GLUT2 (~17 mM glucose), and GLUT4 (~6.6 mM glucose) (Uldry et al. 2002, Thorens and Mueckler, 2010).

Incubation of CHO-TRET1 cells in a 400 mM trehalose solution for 4 h resulted in the intracellular loading of approximately 24 mM sugar. Such a modest amount of trehalose uptake may be due to suboptimal localization of trehalose transporters in the plasma membrane. However, evidence for successful localization of the TRET1 transporter to the plasma membrane is demonstrated by our positive functional data (increase in viability after desiccation and rehydration), and the data provided by Kikawada et al. (2007). Studies by Chen et al. (2001) and

Acker et al. (2002) indicate that to recover reasonable membrane integrity of the cells after drying below 5% of moisture content, the intracellular concentration of trehalose has to be more than 0.2 M (Acker et al. 2002). We were unable to achieve this level of intracellular trehalose in the present study, which could be due to low expression levels of this insect TRET1 in our mammalian cells. Studies are currently underway to optimize the expression of trehalose transporters, which will include modification of the TRET1 DNA sequence to correct for mammalian codon bias.

The process of loading trehalose with TRET1 did not appear to have any adverse effect on the viability of the cells (Fig. 2B). Some procedures for loading sugar, including activation of native channels, permeabilisation of the cell membrane by porating agents and microinjection, have been known to reduce pre-desiccation viability of the cells (Elliott et al., 2006; Eroglu et al. 2000; Eroglu et al., 2003). Clearly, the absence of any adverse loading effect is an advantage of using the TRET1 transporter.

Ma et al. (2005) utilized endocytic loading of trehalose to improve desiccation tolerance of 293H human embryonic kidney cells. When air-dried to 3.5 gH_2O/gdw the study reported cell viabilities of ~70% in presence of 40 mM intracellular trehalose and ~45% if cells were not loaded with the sugar. CHO cells that were incubated for 4 h at 400 mM trehalose presumably also show some endocytotic uptake of the sugar. However, desiccated to a moisture content of 4.32 ± 0.73 gH_2O/gdw membrane integrity increased from ~50% in CHO cells to over 70% in CHO-TRET1 cells. This indicates a clear advantage of using a transporter-mediated trehalose loading technique for this cell type. Cell type specific sensitivities to desiccation, different drying conditions, and the amount of intracellular trehalose may be some of the factors responsible for the differences in desiccation response among cell types. While membrane integrity of cells is an

early indicator of cell survival, some of the cellular damages resulting from desiccation stress can take hours to days to manifest themselves. Similar to other studies (e.g., Acker et al. 2002), we performed long-term growth experiments to investigate proliferation capacities of CHO-TRET1 and CHO cells after desiccation (Fig. 5). Intracellular trehalose introduced by TRET1 improves long-term growth and survival of CHO-TRET1 cells compared to wild-type CHO cells (Fig. 6).

It is important to note that cellular protection by trehalose against damage incurred during desiccation is not the only mechanism at the disposal of anhydrobiotic organisms. Polypeptides such as late embryogenesis abundant (LEA) proteins are used by many anhydrobiotic organisms (Browne et al. 2002, Hand et al. 2007, Chakrabortee et al. 2007; Hand et al., 2011). It has been suggested that LEA proteins work alongside trehalose in anhydrobiotic organisms, and both may be useful for improving desiccation tolerance in mammalian cells (Iturriaga, 2008).

Conclusion

Protection of cells with intracellular trehalose should be an integral part of any dry preservation strategy. Our study demonstrates that loading trehalose into cells with TRET1 improves survival of CHO cells during desiccation.

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List of Figures:

Figure 1: Micrograph of CHO cells (WT and TRET1). The GFP tagged transporters are visible in fluorescence micrograph (wavelength 490nm). The bars in image represent 100 μm.

Figure 2: (A) TRET1 allows CHO cells to increase intracellular trehalose concentration. The intracellular concentration of trehalose was quantified using HPLC. Both transfected and wild-type CHO cells were incubated in cell culture medium containing 100, 250 and 400 mM trehalose for 1, 2 and 4 h. Each value is the mean +/- SD ($n = 3$). (B) Membrane integrity and intracellular trehalose concentration in CHO-TRET1 cells plotted against incubation time. CHO-TRET1 cells were incubated at 37°C in cell culture medium having 100-400 mM trehalose for 0 - 4 h. Error bars are removed for clarity.

Figure 3: Kinetics analysis of zero-trans activity of TRET1 transporter for trehalose in CHO cells. CHO cells expressing TRET1 were incubated in various concentrations of trehalose and the uptake data were fitted to the Michaelis–Menten and Eadie–Hofstee (Inset) equations. Apparent K_m and V_{max} were calculated by nonlinear approximation. Each value is the mean ± SE ($n = 3$).

Figure 4: The viability response of CHO-TRET1 cells (incubated for 4 h in 400mM extracellular trehalose solution) compared to CHO cells (without trehalose transporter) dried to different levels of moisture contents. Viability assessment was performed 45 minutes post-rehydration.

Figure 5: Survival after drying at day 1, 3 and 7 expressed as fractional increase in number of cells in culture for CHO-TRET1 and CHO samples previously dried to the indicated moisture contents. Both CHO-TRET1 and CHO cells were incubated with trehalose before drying. Non-

desiccated control cells (labeled 'C') are depicted with open symbols. Error bars represent ± SE ($n = 7$).

Figure 6: The colony forming abilities of previously desiccated cell samples incubated under normal culture conditions. Samples (A), (B) and (C) were dried to 4.32 ± 0.73, 6.16 ± 0.33 and 8.12 ± 0.52 gH_2O/gdw, respectively, and colony forming units (CFUs) were quantified for each of the conditions.

Figure 1:

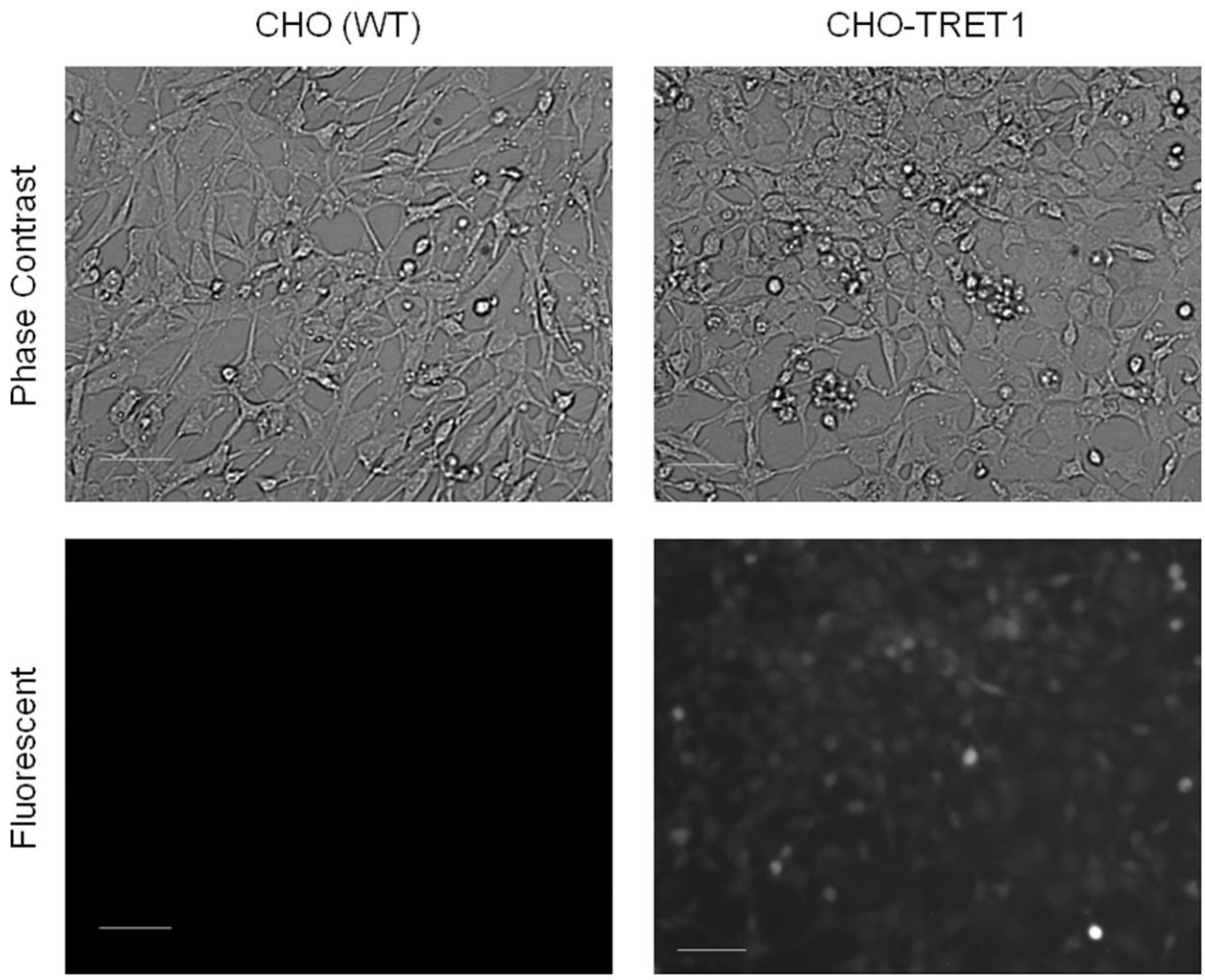


Figure 2:

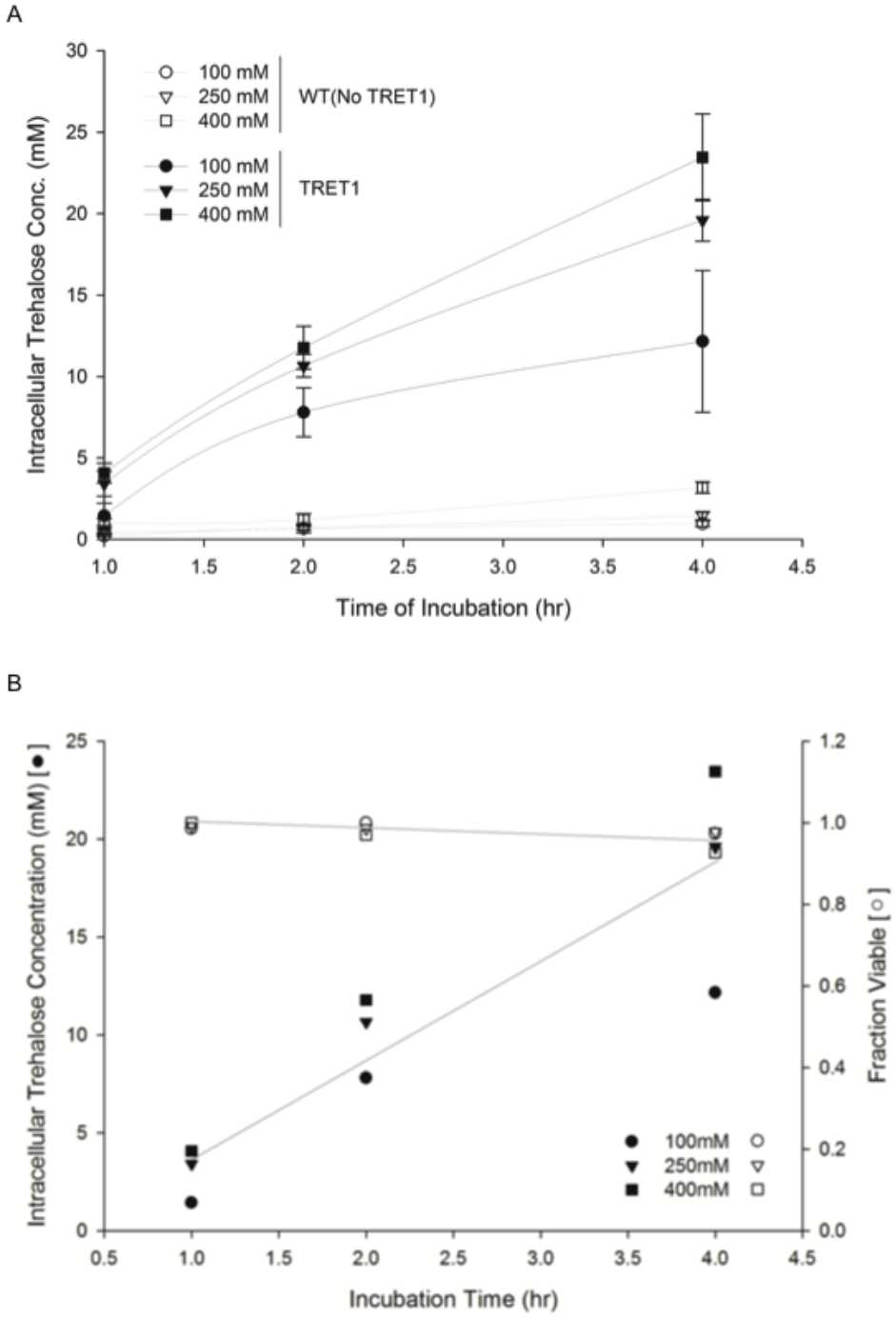

A
100 mM
250 mM
400 mM
WT(No TRET1)
100 mM
250 mM
400 mM
TRET1
Intracellular Trehalose Conc. (mM)
Time of Incubation (hr)
B
Intracellular Trehalose Concentration (mM) [●]
Fraction Viable [○]
100mM
250mM
400mM
Incubation Time (hr)

Figure 3:

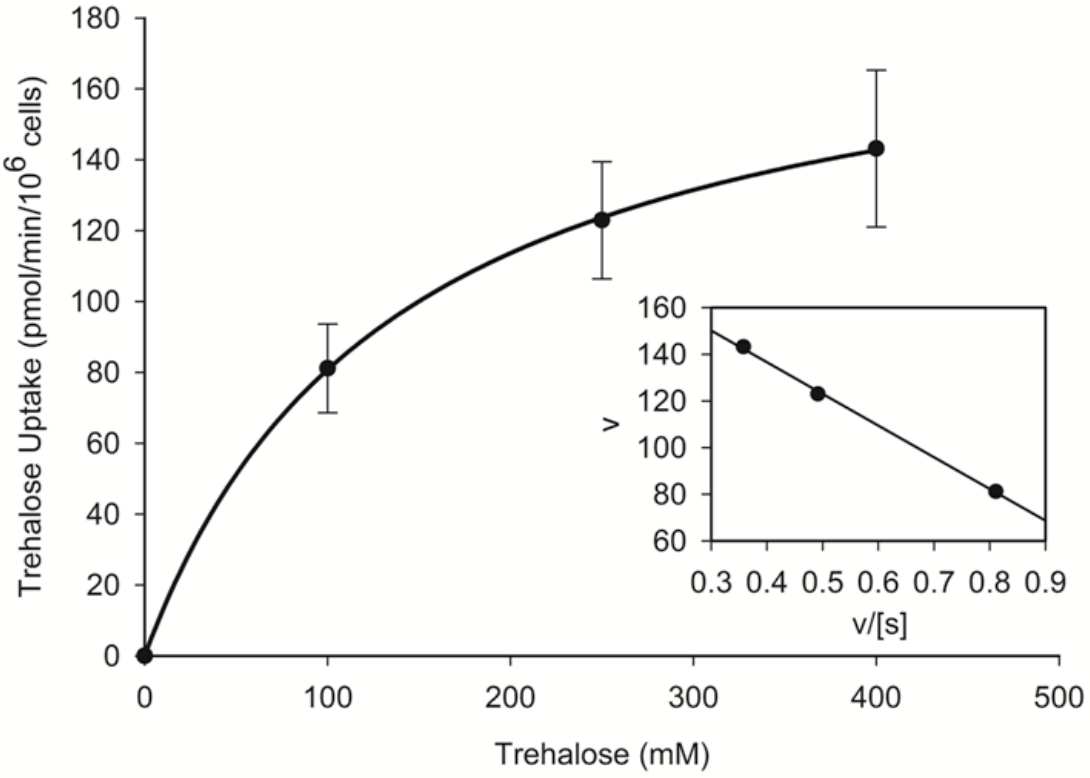

Trehalose Uptake (pmol/min/10^6 cells)
180
160
140
120
100
80
60
40
20
0
0
100
200
300
400
500
Trehalose (mM)
v
160
140
120
100
80
60
0.3 0.4 0.5 0.6 0.7 0.8 0.9
v/[s]

Figure 4:

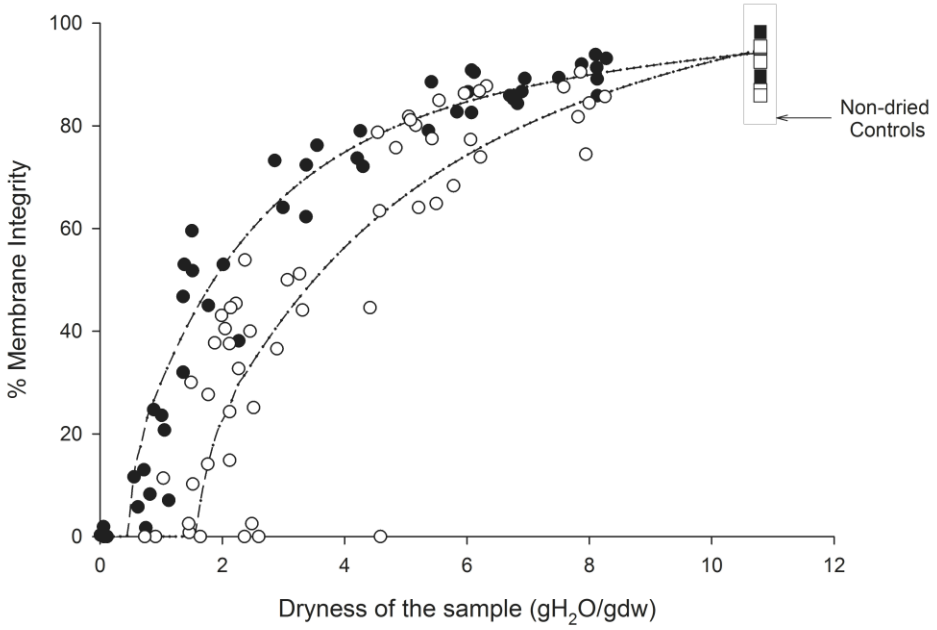

100
80
60
40
20
0
% Membrane Integrity
0
2
4
6
8
10
12
Dryness of the sample (gH2O/gdw)
Non-dried
Controls

Figure 5:

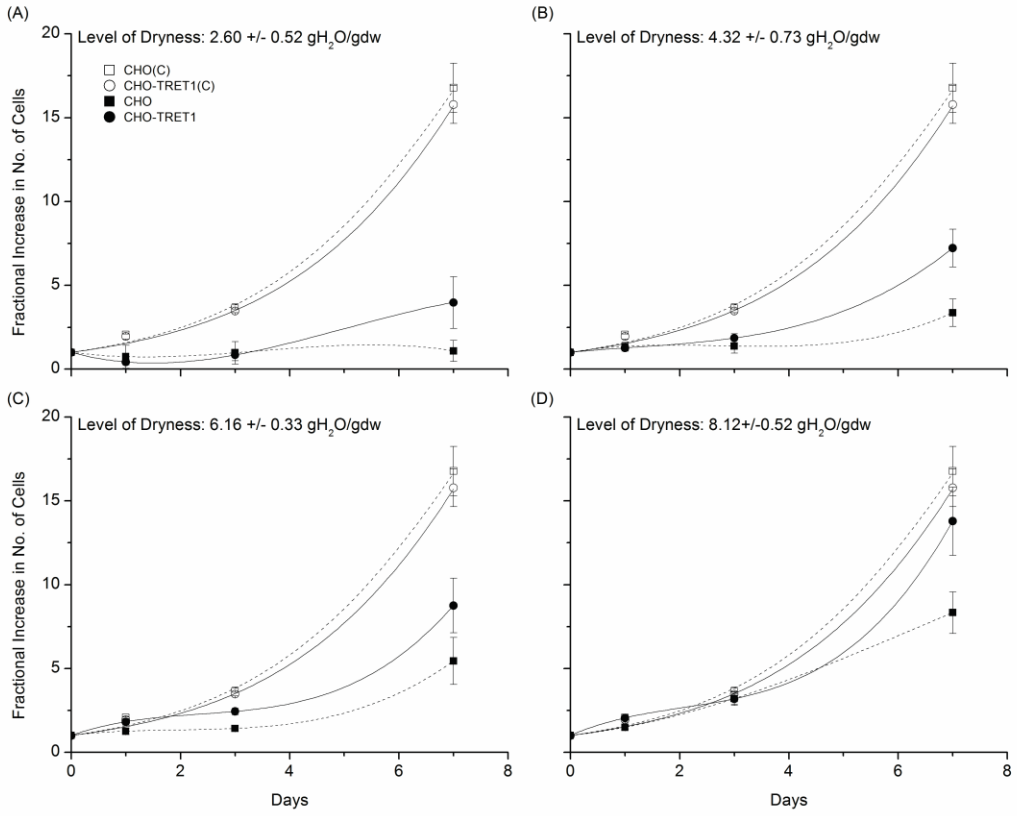
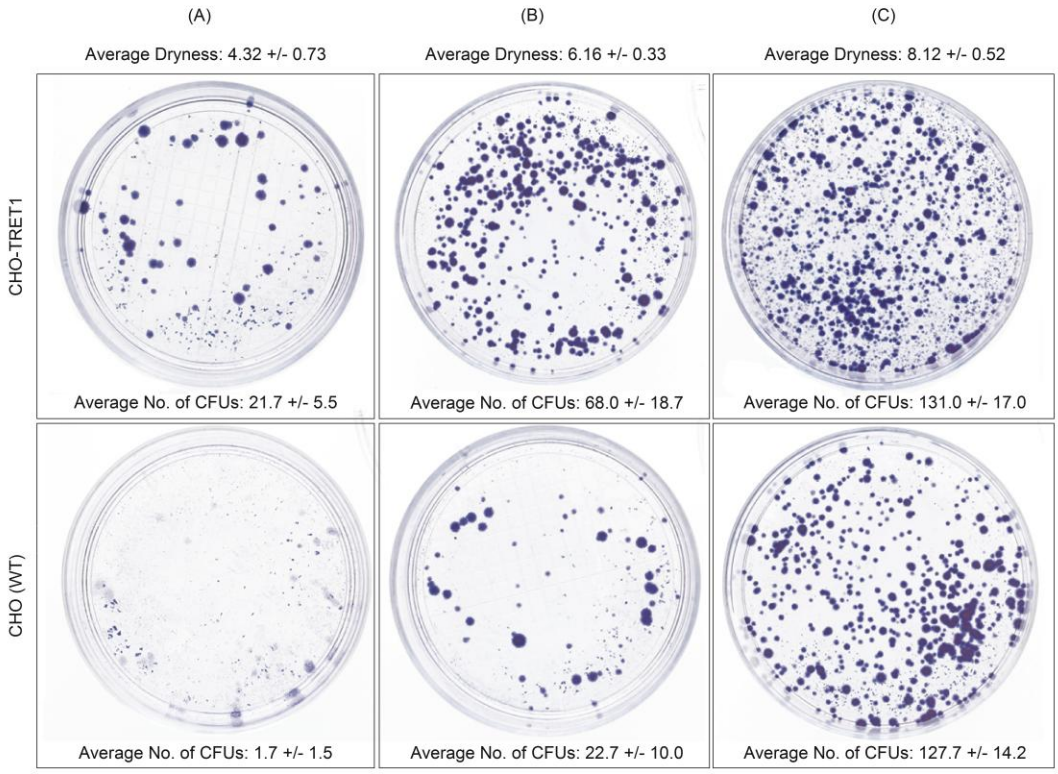

(A)
Level of Dryness: 2.60 +/- 0.52 gH2O/gdw
CHO(C)
CHO-TRET1(C)
CHO
CHO-TRET1
Fractional Increase in No. of Cells
(B)
Level of Dryness: 4.32 +/- 0.73 gH2O/gdw
(C)
Level of Dryness: 6.16 +/- 0.33 gH2O/gdw
Fractional Increase in No. of Cells
Days
(D)
Level of Dryness: 8.12+/-0.52 gH2O/gdw
Days

Figure 6:


(A)
(B)
(C)
Average Dryness: 4.32 +/- 0.73
Average Dryness: 6.16 +/- 0.33
Average Dryness: 8.12 +/- 0.52
CHO-TRET1
CHO (WT)
Average No. of CFUs: 21.7 +/- 5.5
Average No. of CFUs: 68.0 +/- 18.7
Average No. of CFUs: 131.0 +/- 17.0
Average No. of CFUs: 1.7 +/- 1.5
Average No. of CFUs: 22.7 +/- 10.0
Average No. of CFUs: 127.7 +/- 14.2