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Genetic suppression of cryoprotectant toxicity

James R. Cypser[a], Wallace S. Chick[b,c], Gregory M. Fahy[d], Garrett J. Schumacher[a], Thomas E. Johnson[a,e,*]

[a] Institute for Behavioral Genetics, University of Colorado Boulder, USA
[b] Department of Cell and Developmental Biology, University of Colorado Denver, Aurora, CO, USA
[c] Charles C. Gates Center for Regenerative Medicine, University of Colorado Denver, Aurora, CO, USA
[d] 21st Century Medicine, Inc., Fontana, CA, USA
[e] Department of Integrative Physiology, University of Colorado Boulder, USA

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ABSTRACT

We report here a new, unbiased forward genetic method that uses transposon-mediated mutagenesis to enable the identification of mutations that confer cryoprotectant toxicity resistance (CTR). Our method is to select for resistance to the toxic effects of M22, a much-studied whole-organ vitrification solution. We report finding and characterizing six mutants that are resistant to M22. These mutants fall into six independent biochemical pathways not previously linked to cryoprotectant toxicity (CT). The genes associated with the mutations were *Gm14005, Myh9, Nrg2, Pura, Fgd2, Pim1, Opa1, Hes1, Hsbp1,* and *Ywhag*. The mechanisms of action of the mutations remain unknown, but two of the mutants involve MYC signaling, which was previously implicated in CT. Several of the mutants may up-regulate cellular stress defense pathways. Several of the M22-resistant mutants were also resistant to dimethyl sulfoxide (Me_2SO), and many of the mutants showed significantly improved survival after freezing and thawing in 10% (v/v) Me_2SO. This new approach to overcoming CT has many advantages over alternative methods such as transcriptomic profiling. Our method directly identifies specific genetic loci that unequivocally affect CT. More generally, our results provide the first direct evidence that CT can be reduced in mammalian cells by specific molecular interventions. Thus, this approach introduces remarkable new opportunities for pharmacological blockade of CT.

1. Introduction

The limited shelf life of donated organs negatively affects the success of human organ transplantation [1,22,35]. Efficient organ cryopreservation could potentially overcome many obstacles to organ transplantation, thereby reducing costs, improving tissue matching, reducing organ wastage, enabling elective scheduling of transplantation, and facilitating tolerance induction. It could also, in combination with the generation of bioartificial organs or xenograft organ sources, enable an end to the organ shortage. There is now good evidence that the banking of large organs is possible [3,5,14,21,24,29,31,42,54,57,60], but the toxicity of the cryoprotective agents needed to preserve certain organs, such as the kidney, appears to be the major limiting factor [30].

Cryoprotectant toxicity (CT) is particularly problematic during cryopreservation by vitrification (ice-free cryopreservation) [16,53], which has been gaining in popularity in recent years [23,27,28,35,45]. Vitrification requires extremely high concentrations of cryoprotectants [17,28], and the higher the concentration, the more likely it is that CT will arise. Further, since whole organs must be cooled relatively slowly [12], this necessitates using relatively high cryoprotectant concentrations [28]. These high concentrations have high viscosity, which means slow introduction by perfusion and consequently relatively long exposure times to intrinsically toxic concentrations of cryoprotectant. Therefore, direct remedies for CT are needed for vitreous organ banking, and should have numerous practical advantages in cryobiology in general.

Here we describe an entirely new way of addressing the problem of CT. First, we created libraries of transposon-mutagenized mouse embryonic stem cells (ESC). We then selected mutant cells that could survive exposure to 9% of full-strength M22 (an organ vitrification solution; [21]) at 37 °C. This approach (illustrated in detail in Fig. 1) allows the cell itself to identify pathways that are crucial for reducing CT. We demonstrate here, for the first time, the existence of genes whose modulation can confer CT resistance (CTR). We also show that the same genes that confer CTR also provide significant protection

* Corresponding author. Institute for Behavioral Genetics, University of Colorado Boulder, USA.
E-mail address: johnsont@colorado.edu (T.E. Johnson).

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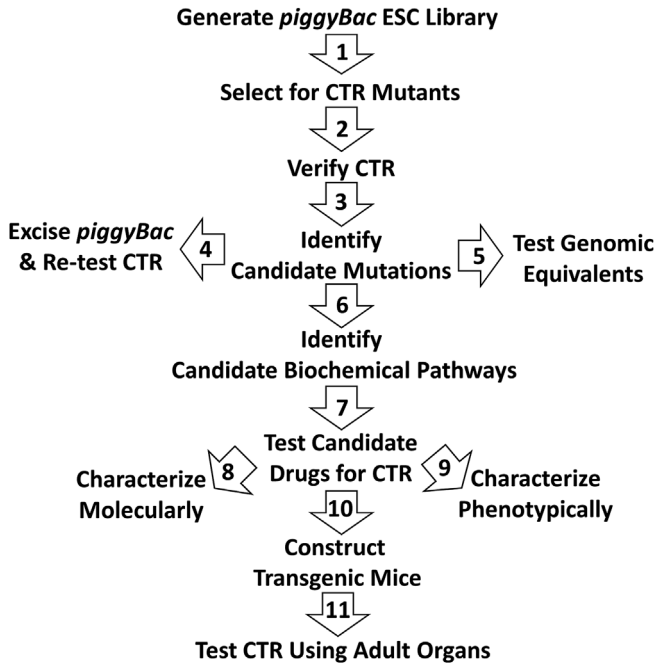

Fig. 1. Overview of strategy for identifying and studying CTR mutants. Mutants are generated and (1) selected for CTR. (2) Mutants are confirmed or rejected. (3) Mutations putatively conferring CTR are identified. (4) Causality is verified by demonstrating that CTR is conferred by other mutations in the candidate gene and is abolished by excising the *pB* transposon and (5) generating genomic equivalents of that mutation. (6) Bioinformatics are used to link the identified mutation to previously-identified pathways. (7) A search is made to identify known drugs that may induce CTR. Candidate drugs are then tested on wild-type cells for efficacy in CTR. Drug effects are characterized (8) molecularly and (9) phenotypically. (10) Ultimately, mice carrying the original mutation are generated and novel improved drugs are developed using a variety of distinct methods, and (11) their organs are tested for CTR.

against freezing injury.

2. Materials and methods

2.1. Solutions

M22 is an 8-component vitrification solution developed by 21st Century Medicine (Fontana, CA) for the cryopreservation of kidneys [21]. M22 was provided for these experiments by 21st Century Medicine. M22 is used at concentrations approaching 9.4 M for the cryopreservation of rabbit kidneys [24]. M22 is composed of 2.8 M Me_2SO, 2.8 M formamide, 2.7 M ethylene glycol, 0.5 M N-methylformamide, 0.3 M 3-methoxy-1,2-propanediol, 2.8% (w/v) (less than 0.006 M) polyvinylpyrrolidone K12, 1% (w/v; less than 0.006 M) polyvinylalcohol-polyvinylacetate copolymer [61], and 2% (w/v; less than 0.03 M) polyglycerol [1]. We refer here to "% M22" (v/v) as the percent of that maximum working concentration (9.4 M) used.

LM5 is the carrier solution and diluent for M22, and was also provided by 21st Century Medicine. It is an aqueous solution of 90 mM glucose, 45 mM mannitol, 45 mM lactose, 28.2 mM KCl, 7.2 mM K_2HPO_4, 5 mM reduced glutathione, 1 mM adenine-HCl, 10 mM $NaHCO_3$, and, when cryoprotectant is absent, 1 mM $CaCl_2$ and 2 mM $MgCl_2$ [21].

Embryonic stem cell (ESC) medium is KnockOut Dulbecco's Modified Eagle's Medium (Gibco, product 10829-018) supplemented with 15% fetal bovine serum (Tissue Culture Biologicals, product 104), GlutaMAX (2 mM L-alanyl-L-glutamine dipeptide in 0.85% NaCl; Gibco product 35050061), 0.1 mM non-essential amino acids (Gibco, product 1140-050), penicillin-streptomycin (25 units/ml, Thermo Fisher, product 15140122), beta-mercaptoethanol (55 μM, Thermo Fisher, product 21985-023) and 1000 unit per ml of leukemia inhibitory factor (AMSBio, product AMS-263-100). DPBS is Dulbecco's phosphate-buffered saline supplemented with calcium and magnesium (Gibco, product 14040-133).

2.2. Cells and cell culture conditions

We generated a mouse ESC line from an F1 hybrid background (C57BL/6J × 129X1/SvJ), referred to here as "C9" [7,8]. For all experiments, cells were thawed and grown for 24 h under standard culture conditions prior to harvesting for experimentation, unless otherwise stated. (We plated 100,000 cells in 5 ml ESC medium, and cultured cells at 37 °C [2].)

2.3. Toxicity of M22 to C9 cells at 2 °C

Cells were trypsinized, divided into equal-volume aliquots of 150,000 cells, pelleted, and supernatants drawn off. The method of M22 addition and removal was modified from Guan et al [36]. All pellets were suspended in 333 μl ESC medium at 2 °C, and brought to 60% M22 by stepwise addition of 2 °C 100% M22 in LM5 to yield 10, 20, 40 and finally 60% M22, at 10-min intervals. After 0, 1, 2, 3, 4 and 8 h of exposure to 60% M22 at 2 °C, single samples were returned from 60% M22 to near 0% M22 using sequential 10-min intervals. The M22 concentration of each tube was adjusted to 30% by adding 600 mM mannitol solution (at 2 °C) in LM5 carrier solution, and then in subsequent intervals, adjusted to 15, 7.5 and 3.75% M22, by adding 300 mM mannitol (at 2 °C) in LM5 [36]. Following return to near 0% M22, each sample was pelleted and cells were resuspended in 5 ml fresh ESC medium and cultured in a T25 flask at 37 °C . At 48 h after the end of each exposure period, the numbers of cells surviving (adherent to the floor of the flask) were counted.

2.4. Mutagenesis and gene identification

Briefly, mouse ESC were mutagenized via random insertion of a *piggyBac* (*pB*) transposon [7,8]. We estimate that the mutant library includes about 42,000 independent mutations (of which approximately 12,000 have been tested). Use of the *pB* transposon allows subsequent excision from the genome (reversal of the mutation) to verify causality. Although most insertions cause loss of function, overexpression has also been observed [8]. The mutant cell lines recovered remain heterozygous for the mutation unless treated further. Full details are available elsewhere [8].

2.5. Selection for CTR

To select mutant cell lines resistant to CT, cells from the library were plated in 15-cm plates at 6×10^6 cells per plate in 9% M22 and cultured at 37 °C for 48 h. Normal ESC medium without M22 was reinstated for 7 days of standard culture, after which six surviving colonies were picked and transferred to fresh individual flasks for recovery and analysis. All *pB* insertion sites were determined by Splinkerette PCR [59]. This allowed identification of the genomic insertion sites associated with CTR.

2.6. Validation of M22-resistant mutants

Mutants initially selected for resistance to M22 (as well as C9 cells used as controls) were re-cultured and seeded at 20,000 cells per well of a 96-well plate in 0–6% M22. After 48 h at 37 °C growth within each well was measured by the MTT assay [48]. The same mutants were also tested for resistance to Me_2SO at concentrations of 0–5%.

2.7. Cryopreservation with 10% Me_2SO

Cells were trypsinized, pelleted, and resuspended at a concentration of 250,000 cells per 250 µl. An equal volume (250 µl) of 2X freezing medium (60% ESC medium, 20% FBS, 20% Me_2SO) was added to each tube, all samples were mixed, promptly placed in a Mr. Frosty cell-cryopreservation container (Thermo Fisher; Waltham, MA) and the container placed in a −80 °C freezer for 24 h. After 24 h, samples were thawed by immersion in a 37 °C water bath, resuspended in 5 ml ESC medium, pelleted, resuspended in a final volume of 500 µl ESC medium and tested for survival using trypan blue and colony formation assays [52,55].

2.8. Viability assays

To test viability using the Trypan Blue assay, 200 µl of each cell suspension in ESC medium was added to 500 µl of 4% (w/v) trypan blue premixed with 300 µl 1X DPBS, mixed thoroughly, and maintained at room temperature for 15 min, followed by scoring of blue (dead) and clear (live) cells.

To test viability using colony formation ability, 300 µl of each cell suspension in ESC medium were plated in 5 ml of ESC medium in a T25 flask and cultured for 48 h, at which time dead cells (not adhered to the flask) were removed by rinsing twice with DPBS. This assay relies on counting colonies by eye to assess viability. To assess reliability, we used two independent estimates provided (blind) by two scorers counting the same sample, and results were compared (Fig. 2; note high value of r^2).

2.9. Statistics

All statistics (*t*-test; proportional survival) were performed in Microsoft Excel. For Trypan Blue assays, all p-values are tests of independent proportions of living cells. For colony formation assays, all p-values are one-way t-tests of the mean number of colonies observed. For MTT assays, all p-values are two-way t-tests of the mean absorbance (representing living cells) at a given concentration.

3. Results

3.1. M22 toxicity in wild-type ESC

Initially we tested the toxicity of the cryoprotectant solution M22 at 60% and 2 °C, in wild-type mouse ESC, to determine reasonable conditions for later mutant selection. We observed an exponential rate of loss that continued until hour 4, after which about 30% of the cells continued to survive for as long as 8 h with little additional toxicity (Fig. 3).

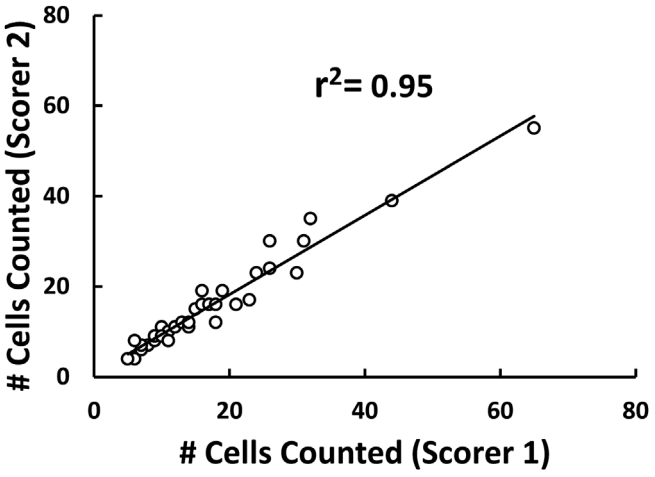

Fig. 2. Independent counts of cell colonies by different scorers.

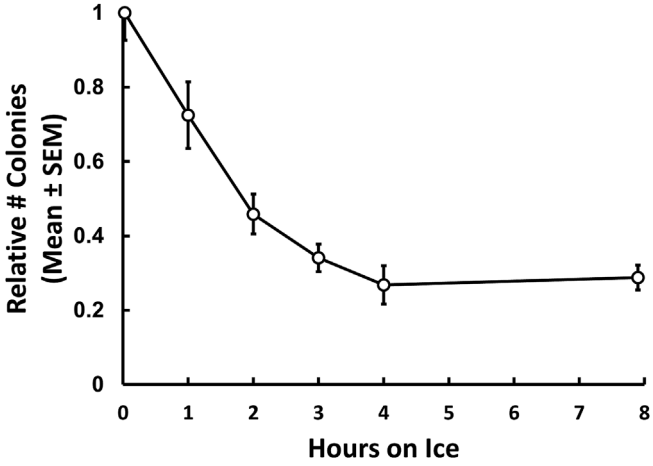

Fig. 3. Survival of mouse C9 cells during exposure to 60% M22 at 2 °C, by the method of Guan et al [36]. Shown are the pooled results of two experiments.

An effective selection of mutants requires conditions that kill nearly all cells (mutant or wild-type), selecting only resistant mutants. We therefore tried increasing the M22 concentration to 80% at 2 °C, which resulted in more cell death, but also resulted in living cells that displayed exceptionally slow growth and abnormal appearance. To avoid dealing with such slow-growing cells, we asked whether exposure of mutant ECSs to lower concentrations (5% or 10%) of M22 for prolonged periods at higher temperatures would result in a smaller population of surviving cells that still displayed reasonable growth rates. We found that selection in 5% M22 permitted too many ESC to survive, while selection in 10% M22 provided no clones capable of further replication (Fig. 4A).

3.2. Identification of M22-resistant mutants

We found that exposure of normal ESC to 9% M22 at 37 °C over seven days resulted in near-complete lethality (Fig. 4B). We next exposed mutated ESC to 9% M22 for 48 h and found nine rare resistant mutant clones. We were able to identify the insertion site of the *pB* transposable element for six of these (Table 1).

Two resistant mutants (M2.1/*Gm14005* and M4.3/*Myh9*) were challenged with 0–6% M22 for 48 h and displayed significantly increased survival compared to the parental wild-type cells (Fig. 5A). Similarly, when mutants M2.1/*Gm14005* and M4.3/*Myh9* were challenged in the same way with Me_2SO, they displayed significantly increased survival (Fig. 5B).

3.3. Resistance of mutants to standard cryopreservation conditions

We next asked whether mutants displaying resistance to M22 under otherwise-normal cell culture conditions would also display greater viability under standard cryopreservation conditions with Me_2SO. Viability was measured by both the trypan blue dye exclusion assay [55] and a colony formation assay (the ability of cells to form colonies adherent to the floor of the tissue culture flask [52]).

Mutant 2.1, containing a *pB* insertion in *Gm14005*, displayed particularly high survival after freezing and thawing as measured by trypan blue exclusion (Fig. 6A–C). In Fig. 6A, mutant M2.1 showed a 12.7-fold increase over control survival (indicated as being outside the range of all other mutants in multiple experiments). Other tested mutants also displayed more moderate but still much higher survival relative to non-mutant controls (improvements of 3–4-fold; Fig. 6B–C). The colony formation assay confirmed that the mutant M2.1/*Gm14005*

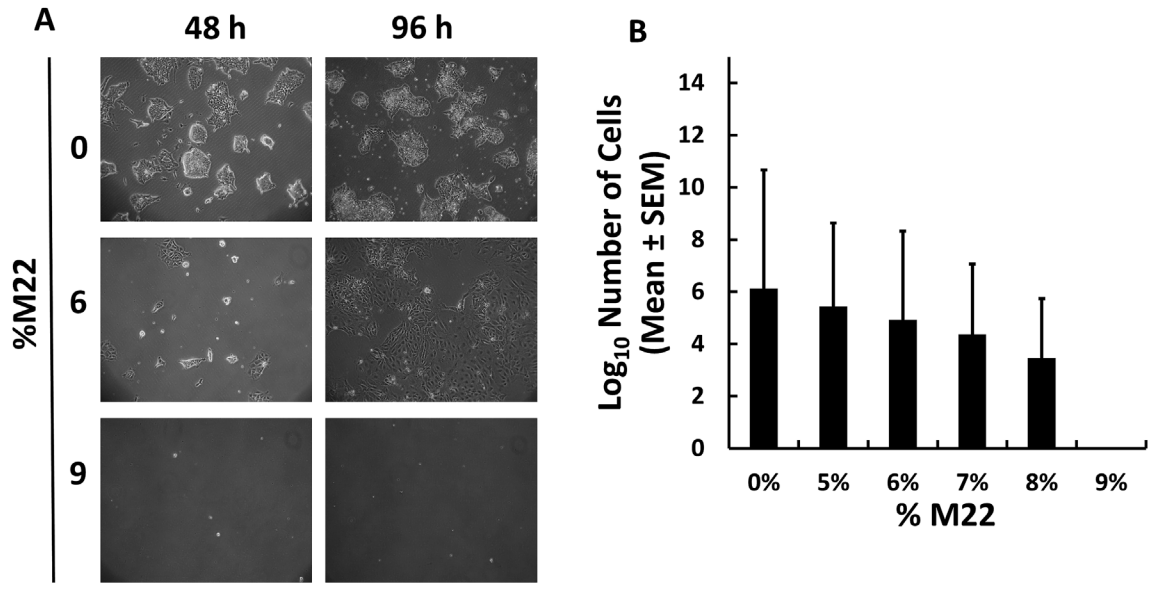

Fig. 4. A. Morphology of C9 cells under various M22 conditions, at 37 °C. Images obtained at 0%, 6%, and 9% M22 after 48 and 96 h of exposure. Note decreasing size and increasingly smooth border of colonies or cells as M22 concentration increases. **B**. Dose-response effects of exposing C9 cells to M22 for 96 h at 37 °C.

Table 1
pB insertion sites in CTR mutants.

Name	Gene	Chromosome	5′ end	3′ end
M2.1	*Gm14005*	2	128377777	128377682
M2.2*	*Nrg2/Pura*	18	36257514	36257419
M3.1*	*Fgd2/Pim1*	17	29460474	29460569
M4.2*	*Opa1/Hes1*	16	29682841	29682936
M4.3	*Myh9*	15	77791364	77791269
M5.1*	*Hsbp1/Ywhag*	5	3280	3375

Sequences identifying genomic locations of *pB* insertions sites associated with resistance to M22. “Start” and “End” indicate the position of each *pB* insertion and the 5′ and 3’ ends of the sequence used in BLAST for alignment. All sequences used in BLAST were 95 base pairs, found in BLAST searches using the Mouse Genomic Plus Transcript (Mouse G + T) database against mouse genome assembly version GRCm38.p6. Asterisks indicate intergenic insertions between the two flanking genes. For exact sequences see Table 2.

displayed unusual resistance (3.5-fold improvement), as did two other mutants (2–3-fold improvement; Fig. 6D–F).

4. Discussion

4.1. Mutations can confer CTR and cryoprotection

To our knowledge, this is the first report of mutations in mammalian cells that confer CTR and resistance to freeze-thaw injury. Although there are genes known to confer protection against freezing and thawing in free-living bacteria [47], genes that modulate similar processes in mammalian cells have not been previously described.

The protective effects of the mutations described here were found to be very dependent upon the nature of the stressor. Survival of the mutants was at best only about 20% higher after the severe treatment of prolonged M22 exposure at 37 °C. Survival was about 60% higher after similar treatment with Me_2SO. However, this translated to survival after a more typical freezing and thawing stress that was up to four times higher than for normal ESC. This greater effect on survival after freezing may occur because freezing and thawing induces injury that may include significant CT caused by freeze-concentration of the cryoprotectant [15,18,19,25]. Injury caused by freeze-concentration is expected to be easier to control than toxicity at 37 °C due to the protective effect of much lower temperatures [13,31,54] and the relatively short exposure times involved in cryopreservation vs. the extreme exposure times tested at 37 °C.

Although mutations that were protective at high temperatures were also protective at low temperatures, the temperature of exposure is expected to affect at least some of the mechanisms of CT [2,19,28]. Therefore, different protective mutations might emerge from different types of selection in future experiments. For example, selection experiments could be performed using shorter exposure times and higher concentrations at either 37 °C or much lower temperatures.

The results presented here indicate that the detrimental effects of cryoprotectant exposure depend on normal biochemical processes and can be inhibited by mutations. In this regard, selection at 37 °C will certainly favor the discovery of pathways that have detrimental effects in the presence of cryoprotectants, since any such pathways will be maximally active at this temperature. However, our observations of a biphasic response to high concentrations of M22 at low temperatures within a genetically homogeneous population (Fig. 3) is also consistent with the possible existence of an adaptive state. This adaptive state may pre-exist in a small subset of cells, or it may be induced by exposure to M22. There is precedent for an adaptive state in the observation that mammalian cells in log phase growth are considerably more resistant to

Table 2
DNA sequence immediately downstream of *pB* transposon insertion.[a]

M2.1	**Gm14005** TTAAGATTCGGGTATACAAAACCCCTTTTTCTTTCTTTCTTCCCGCCTTCCTTTGAGTAATTGCTTTCTTCCACAGTTACATAATTAGTCGCTGC
M2.2*	**Nrg2/Pura** TTAATCATTTTGAGATGTGGGATTTTGTTTGCTATCAACTCAGTTCACATTTTGTGTACTACAAAGTTTYCTTAGTAGCTCTTATTTACTGTGGT
M3.1*	**Fgd2/Pim1** TTAAGCTCCCCTGTTCTTAGGAAACCACAGAGATTCCAGAAGGACTAGCTCAGATATTGTGCCTTTCCCTGACTTAATAGGTCTCCTTCTTAAAA
M4.2*	**Opa1/Hes1** TTAAGATGTTCACCTGTTCCTGAGCTTGCAATTTTATTAAGAGGGAATTTGAAAATTCAGGATTTCTTCGATGGAAAATATGGGGTTTTTAAGAC
M4.3	**Myh9** TTAAGCAGACACGGCTCTCCGAGTGTGCGAGCTGCACGCTTAGTCCAGCAGTCTCTTTGAAGGCTGGAGGCGAGGTGGTTGGCTTTGCCCTGCTT
M5.1*	**Hsbp1/Ywhag** TTAATAATGTCTGGTGTTTGTCATGGTGAAACTATAACTATCGCAGTGCAGGAGAACGCGCGGACGTTCTCAGAACGCGCCTCTGTGCTTTCTTG

[a] Asterisks indicate intergenic insertions between the two flanking genes. In each case the 5′ insertion site of the transposon is after the TTAA lead sequence.

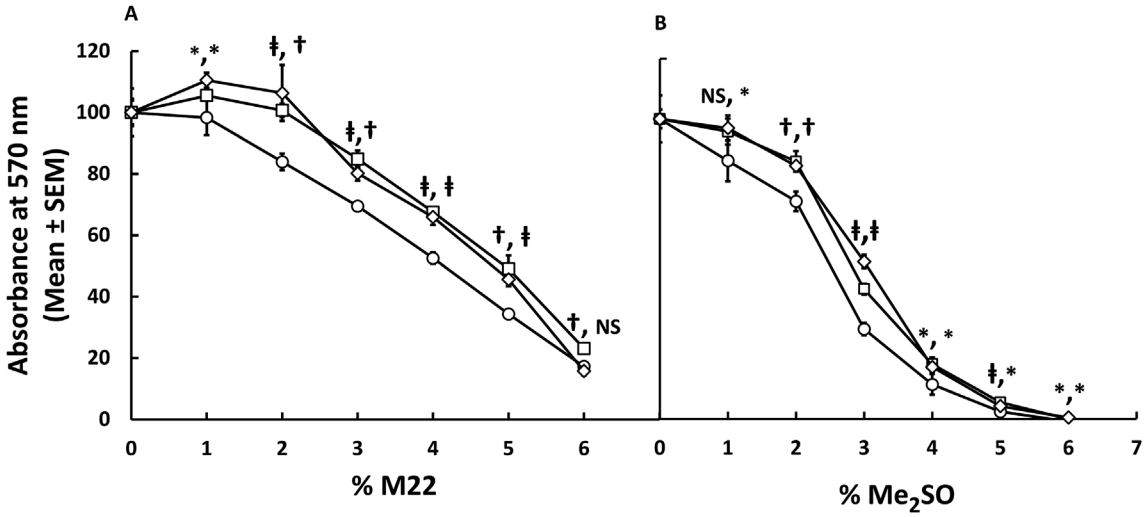

Fig. 5. A. Survival (measured by the MTT assay) of controls (circles), mutant M2.1/*Gm14005* (squares), and mutant M4.3/*Myh9* (diamonds), with 0–6% M22 at 2 °C for 48 h. Mutant measurements are normalized to controls. All p-values are independent, one-tailed t-tests of means: *, $p < 0.05$; †, $p < 0.01$; ‡, $p < 0.001$. **B**. Survival (measured by the MTT assay) of mutants M2.1/*Gm14005* and M4.3/*Myh9* exposed to 0–5% (w/v) Me_2SO at 37 °C for 48 h. Symbols represent mutants and significance levels as in **A**.

freezing and thawing than are stationary phase cells [33]. Further, in bacteria [47] and yeast [44,50], freeze-thaw survival is also dependent on cell cycle stage and metabolic conditions, including starvation and other applied stresses [50]. Such a protective state would support the possibility of deliberately altering gene expression to significantly reduce CT and open up the prospect of discovering additional protective mechanisms of clinical relevance.

4.2. Identified mutants suggest multiple possible pathways modulate CTR

The specific mutated gene providing the strongest protection in our freeze-thaw assays, *Gm14005*, is poorly characterized. It is most likely transcribed only into long non-coding RNA (lncRNA) species (see http://www.informatics.jax.org/sequence/marker/MGI:3652191?provider=RefSeq.). lncRNAs have regulatory effects [46], so the identification of *Gm14005* points to previously unknown mechanisms, both for CTR and for freezing injury.

The strongest mutation for abrogating toxicity at 37 °C, M4.3/*Myh9*, encodes a non-muscle myosin protein involved in cell spreading (see http://www.genecards.org/cgi-bin/carddisp.pl?gene=MYH9). The mechanism of action is not clear. Our colony formation assay requires adhesion of the ESC to their culture dishes, and adhesion requires myosin-related function. It is not clear why reducing or knocking out expression of a single myosin gene would improve that function, or

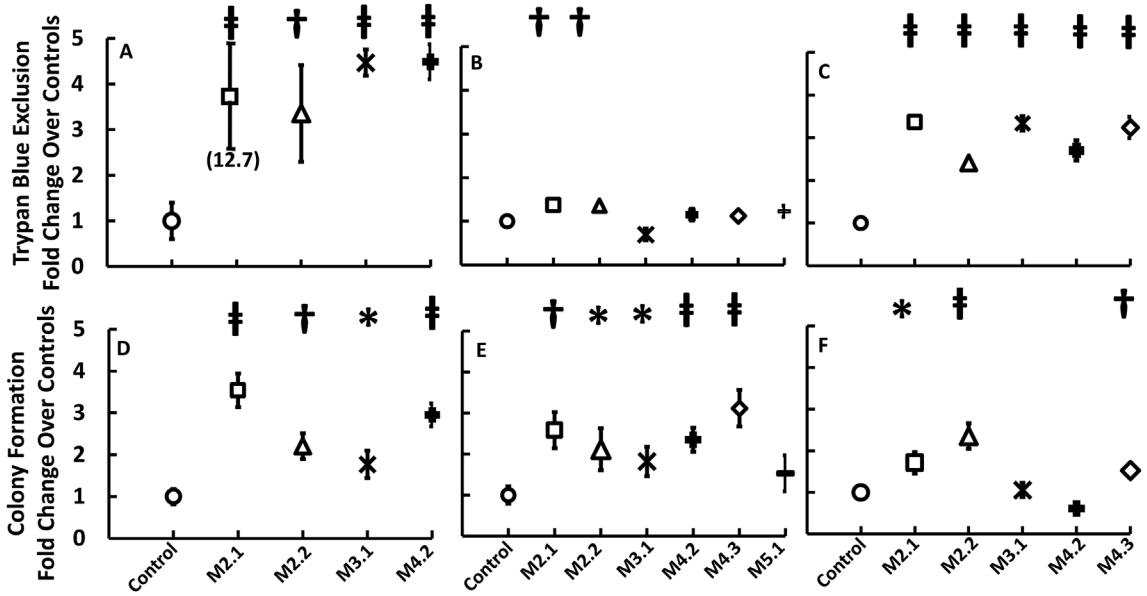

Fig. 6. Mutant viability after 5-min exposure to 10% (v/v) Me_2SO at 37 °C, followed by freezing at −80 °C, storage for 24 h, and thawing. Mutants represented include M2.1/Gm14005 (squares), M2.2/*Nrg2/Pura* (triangles), M3.1/*Fgd2/Pim1* ("X"), M4.2/*Opa1/Hes1* ("+"), M4.3/*Myh9* (diamonds), and M5.1/*Hsbp1/Ywhag* (horizontal bars). **A-C**: Trypan blue assay: Mutant proportions alive are normalized to control proportion alive. In the first experiment shown (panel 6A) mutant M2.1/*Gm14005* displayed resistance 12.7-fold more than controls; that exceptional value is annotated within the graph. All other fold-values greater than controls were less than 5. Controls are represented by circles. All p-values are tests of independent proportions alive: *, $p < 0.05$; †, $p < 0.01$; ‡, $p < 0.001$. **D-F**: Colony formation assay: Counts of mutant colonies are normalized to control counts. All p-values are independent, one-tailed t-tests of means. Symbols representing mutants and significance levels are as in **A-C**.

what other myosin-related function may be key, but interestingly, the reduced freeze-thaw survival of mammalian cells in stationary phase vs logarithmic growth phase is reversed by trypsinization [33]. Other studies have indicated that impairment of this non-muscle myosin also protects against oxidative damage [43]. However, so far we have not observed other CTR mutants with evident roles in modulating oxidative stress.

The other four identified locations of *pB* insertion fall between known genes. For example, the mutations in clones M2.2 and M3.1 fall between *Nrg2* and *Pura* and between *Fgd2* and *Pim1*, respectively. It is not obvious why modification of NRG2 or FGD2 function might reduce CT. However, the functions of PURA and PIM1 may be related to previous observations of the transcriptomic response to CT in rat liver slices exposed to two M22-like vitrification solutions [37]. One of the most interesting findings was a 40% increase in expression of the transcript for MYC [37]. MYC is a regulator of ribosome biogenesis and protein synthesis, and might have been upregulated because of its role in facilitating the replacement of proteins chemically damaged or denatured by exposure to cryoprotectant [27,28]. It is tempting to think that, in our experiments, PURA modulation may better activate MYC (http://www.uniprot.org/uniprot/P42669) and PIM1 modulation may better stabilize MYC (http://www.uniprot.org/uniprot/P06803). MYC is, of course, also associated with cell division [6], which might relate to the observation that rapidly dividing mammalian cells are protected against freezing injury [33]. In principle, however, the *Myc* upregulation observed in the transcriptomics study could be maladaptive rather than protective, given that protein synthesis in the presence of previously denatured proteins can lead to apoptosis or other adverse events via the unfolded protein response (endoplasmic reticulum stress) [34,41]. Furthermore, mice heterozygous for loss of MYC function display at least an 11% increase in longevity without significant difference in cancer as a cause of death, as well as an increased healthspan [38]. Improvements in these traits are generally associated with increased resistance to manifold types of stress [11,40], which indicates that MYC may inhibit stress responses. In any case, it is notable that two studies based on entirely different methods for elucidating mechanisms of CT have both pointed to a common gene, and it will be interesting to investigate the implications.

One of the two genes flanking the M4.2 mutation (*Opa1*) modulates the mitochondrial permeability transition pore, which plays a key role in apoptosis [51]. Possibly, the mechanism of protection is blockade of apoptosis long enough for surviving cells to repair sub-lethal injury, although apoptosis was not upregulated in either of two transcriptomics studies of CT [10,37].

The *pB* insertion site of the last resistant clone, M5.1, is flanked by two genes (*Hsbp1* and *Ywhag*) that may modulate stress resistance. The former may downregulate the overall stress response [56], and the latter encodes a 14-3-3 protein [39]. 14-3-3 proteins have been shown to retain the FOXO3 protein in the cytoplasm, a state associated with reduced stress resistance [4,49].

The studies reported here provisionally establish causality between the described mutations and protection against injury, but an additional step should be taken to completely prove this causality. Our choice of the *pB* transposon-to mutagenize ESC will enable us to excise the transposon in future studies. If doing so restores the wild-type cell susceptibility to cryoprotectant exposure and cryopreservation, causality between the mutation and the observed protective effects will be beyond doubt. Validation of the resistance of these (or newly-generated) mutants could also be supported by generation and testing of similar mutations using CRISPR-Cas9 or other genomic alterations.

4.3. Our model probably selects for resistance to non-specific CT

The maximum tolerated concentration of M22 at 37 °C was about 6% (v/v) M22, or about 4.5% (w/v) M22 cryoprotectants, which translates to about 0.65 M cryoprotectant, and 1.6% (w/v) Me_2SO. The maximum tolerated concentration of Me_2SO by itself was 5% (v/v), which equals about 0.71 M, or 4.5% (w/v) Me_2SO. Therefore, at 37 °C, ESC respond to the total cryoprotectant concentration roughly equally on a molar basis, and not to the concentration of Me_2SO or any other specific component. This suggests that the effects of cryoprotectants when tested as described are not chemically specific, even though in principle, metabolism of some of the cryoprotectants might be possible, and Me_2SO might be able to react with –SH groups [58]. Non-specific cryoprotectant toxicity is a desirable standard for comparison between different cryoprotectant formulations and is believed to be the type of toxicity that is most relevant to organ cryopreservation by vitrification [20,26].

Me_2SO is known to be a differentiating agent when applied under the right conditions [9,32], but we do not think differentiation of ESC modulated either CTR or cryopreservation injury. First, our mutant cells behave and appear like ordinary ESC, rather than as differentiated cells, and none of the mutations we identified are expected to reverse or induce differentiation. Second, it is not obvious that differentiating ESC would make them either more or less susceptible to cell death caused by CT or cryopreservation, and it is cell death that we selected against. Finally, mutants displaying resistance to M22 also resisted Me_2SO and cryopreservation in Me_2SO, but M22 and Me_2SO would presumably not have the same differentiating effects.

4.4. Identified mutant cell lines may be convertible into resistant whole mouse strains

Mutated ESC have been grown into adult mice displaying the same phenotype as the mutant ESC [7]. This suggests that CTR may also be transmissible to whole animals derived from CT-resistant ESC. The cells, tissues, and organs of such animals might serve as excellent models for further efforts to ablate CT. Furthermore, if acceptably mutated large animal ESC can be developed into adult tissues that resist cryoprotectant toxicity and cryopreservation injury, it may be possible to create unlimited supplies of replacement organs bearing such mutations for human transplantation.

4.5. Resistant mutants can be used to identify extant drugs with the same function

Our mutants' utility for normal cryopreservation lies in the possibility of using drugs to mimic the effects of these mutations. Given MYC's relationship to cancer, many extant strategies for inhibiting MYC to inhibit cancer may be repurposed to inhibit CT and cryopreservation injury. If a conventional drug implicated by a given mutant is not available, other strategies such as microRNA or CRISPR-Cas9 technology might be used to create novel drugs with the same therapeutic effects. Therefore, our novel fundamental discovery that inhibiting gene expression can inhibit CT and cryopreservation injury has implications for the cryopreservation of many cell types, tissues, and even whole organs.

4.6. The advantage of forward genetics for elucidating and countering mechanisms of CT

Cryoprotectants interact with literally every molecule in the cell, and in the case of organs, with every cell type in the organ. This makes it difficult to elucidate mechanisms of CT and find remedies for it. There are now two microarray studies of the transcriptional responses of organ components to cryoprotectant exposure [10,37], which appropriately apply a global method of investigation to understanding the global influence of cryoprotectants. However, the mechanistic and therapeutic inferences that can be made from such studies are limited. Invariably, many pathways are found to be up- or down-regulated, and it can be difficult to determine whether specific pathway changes are relevant to the essence of CT or are merely secondary effects. It is also

difficult to know in many cases whether observed changes in gene expression are therapeutic (compensatory) or pathologic. For example, as mentioned above, one transcriptomic study [[3,37]] found up-regulation of MYC after CT, which was interpreted as a possible compensatory response, but in the present study, CTR was associated with a mutation that nominally would be expected to reduce rather than to increase MYC expression. *A priori*, it is impossible to know whether activation of MYC is protective or detrimental, but since we know our mutant is protective, we can answer the question of benefit vs harm directly, simply by determining in a follow-up study whether MYC expression is up- or down-regulated in our MYC-related mutants. In principle, a finding of a change in MYC expression in transcriptomic studies can be followed up with drug studies to see whether a drug that opposes the observed change in transcription is beneficial or detrimental, but in transcriptomic studies, there may be 100 candidate genes of uncertain relevance to so investigate [10,37], rather than just one gene of certain relevance for any given mutant discovered using forward genetics.

In summary, our results open up new opportunities for improving both freeze-thaw cellular viability and the viability of multicellular systems after cryopreservation by vitrification. By challenging cells under the extreme but experimentally convenient conditions of high temperature and long exposure time, we were able to identify protective mutations providing in some cases profound improvements in survival under conditions that are actually of interest to the cryobiologist. It will be interesting to see whether future studies uncover additional protective effects and to see whether combining CTR mutations and/or equivalent drugs can augment protection.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cryobiol.2018.11.003.

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