

# Molecular approaches for improving desiccation tolerance: insights from the brine shrimp *Artemia franciscana*

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## Abstract

**Main conclusion** We have evaluated the endogenous expression and molecular properties of selected Group 3 LEA proteins from *Artemia franciscana*, and the capacity of selected Groups 1 and 3 proteins transfected into various desiccation-sensitive cell lines to improve tolerance to drying.

Organisms inhabiting both aquatic and terrestrial ecosystems frequently are confronted with the problem of water loss for multiple reasons—exposure to hypersalinity, evaporative water loss, and restriction of intracellular water due to freezing of extracellular fluids. Seasonal desiccation can become severe and lead to the production of tolerant propagules and entry into the state of anhydrobiosis at various stages of the life cycle. Such is the case for gastrula-stage embryos of the brine shrimp, *Artemia franciscana*. Physiological and biochemical responses to desiccation are central for survival and are multifaceted. This review will evaluate the impact of multiple late embryogenesis abundant proteins originating from *A. franciscana*, together with the non-reducing sugar trehalose, on prevention of desiccation damage at multiple levels of biological organization. Survivorship of desiccation-

sensitive cells during water stress can be improved by use of the above protective agents, coupled to metabolic preconditioning and rapid cell drying. However, obtaining long-term stability of cells in the dried state at room temperature has not been accomplished and will require continued efforts on both the physicochemical and biological fronts.

**Keywords** Anhydrobiosis · Diapause · Intrinsically disordered proteins · Late embryogenesis abundant proteins · Trehalose

## Abbreviations

LEA	Late embryogenesis abundant
ROS	Reactive oxygen species
$T_g$	Glass transition temperature
GFP	Green fluorescent protein
TFE	Trifluoroethanol
PFK	Phosphofructokinase
CS	Citrate synthase
LDH	Lactate dehydrogenase
AMPK	AMP activated protein kinase
AICAR	5-Amino-4-imidazole carboxamide ribonucleoside
ZMP	5-Amino-4-imidazole carboxamide ribonucleoside monophosphate

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## Introduction

Water resources are a major driving force in terrestrial ecology and can shift species interactions, and climate change is expected to greatly alter the distribution of this critical resource (McCluney et al. 2012). Moreover, water availability has pronounced influences on animal activity,

distribution patterns, and species richness (Chown et al. 2011). Drying because of evaporative water loss is the most common mechanism for dehydration, although during winter in temperate regions freezing can also occur, which reduces the liquid water in extracellular fluids and can lead to intracellular dehydration in multicellular organisms. Behavioral, physiological and biochemical responses are all important for resisting and/or tolerating water loss in organisms. Identifying and understanding the mechanisms present in organisms whose evolutionary history has provided the capacity for natural tolerance to drying and freezing will inform us about fundamental ways by which water limitation can be survived by cells and tissues.

Animals from four different phyla, selected land plants, certain fungi and bacteria survive severe desiccation for extended periods (Crowe and Clegg 1978), a phenomenon known as anhydrobiosis, or ‘life without water’ (Keilin 1959; Crowe and Clegg 1973; Crowe et al. 1997). Multiple molecular components may contribute to the intracellular conditions required for successful desiccation in nature, and metabolic preconditioning for embryonic stages (Hand et al. 2011a) may be important as well. Some organisms contain solutes, such as trehalose, that can stabilize biological structures during severe desiccation (Xie and Timasheff 1997; Crowe et al. 1997, 2005; Yancey 2005). More recent investigations underscore the contributions of various protective proteins during drying, including stress proteins (Clegg 2011), anhydrin and late embryogenesis abundant (LEA) proteins (Tunnacliffe and Wise 2007; Hand et al. 2011b), the latter two being intrinsically disordered proteins (Uversky and Dunker 2010; Chakrabortee et al. 2012). Polyamine utilization, glyoxalase-dependent detoxification, lipid desaturation pathways, and defenses against reactive oxygen species (ROS) also have been implicated in tolerance to severe desiccation (Erkut et al. 2013).

LEA proteins were first identified in plant seeds (Dure et al. 1981) and more recently have been reported in several animal groups, including nematodes (Solomon et al. 2000; Browne et al. 2002), rotifers (Tunnacliffe et al. 2005), insects (Kikawada et al. 2006), crustaceans (Hand et al. 2007), springtails (Clark et al. 2007), and tardigrades (Forster et al. 2009) (for more extensive literature reviews, please see Tunnacliffe and Wise 2007; Hand et al. 2011b). Multiple LEA proteins are commonly found in a given species (Warner et al. 2010, 2012; Candat et al. 2014), which may be explained in part by targeting to different cellular locations. An organism’s expression levels of LEA protein and mRNA are closely tied to its capacity for desiccation tolerance (e.g., Browne et al. 2004; Gal et al. 2004; Hand et al. 2007; Menze et al. 2009). Indeed the knockdown of Group1 LEA proteins reduce survival for embryos of *Artemia franciscana* after desiccation and

freezing (Toxopeus et al. 2014). LEA proteins are highly hydrophilic and generally unstructured, with a high percentage of random coils in aqueous solution. Secondary structures, such as  $\alpha$  helices,  $\beta$  sheets, and hairpin loops, form as water is removed (Hand et al. 2011b). Multiple functions have been proposed for LEA proteins with varying degrees of experimental support, including action as a “molecular shield” to sterically reduce aggregation of denatured proteins (Chakrabortee et al. 2012), stabilization of target proteins via chaperone-like activity, protection of biological membranes (Tolletter et al. 2007, 2010), stabilization of vitrified sugar glasses by increasing the glass-transition temperature ( $T_g$ ) (Shimizu et al. 2010), and sequestration of divalent ions (cf. Tunnacliffe and Wise 2007; Hand et al. 2011b). Finally, LEA proteins and trehalose display synergistic interactions in their abilities to protect target proteins during drying (Goyal et al. 2005), a phenomenon also reported for stress proteins and trehalose during drying of nucleated cells (Ma et al. 2005). The temporal interplay between trehalose and heat shock proteins in dried yeast cells emphasizes the complementary roles played in some cases by these two agents (Tapia and Koshland 2014).

For several years, our laboratories have investigated the desiccation-protective mechanisms of LEA proteins and intracellular organic solutes that are found endogenously in anhydrobiotic embryos of the brine shrimp, *Artemia franciscana*. *A. franciscana* is the only known animal species that naturally expresses LEA proteins from three different classification groups (Groups 1, 3, 6; for classification scheme, see Wise 2003). In this review, we will evaluate the endogenous expression and molecular properties of selected Group 3 LEA proteins from *A. franciscana*, and the capacity of selected Groups 1 and 3 proteins transfected into various desiccation-sensitive cell lines to improve tolerance to drying.

### Endogenous expression of Group 3 LEA proteins in *A. franciscana*

Three new LEA proteins, predicted to be mitochondrial-targeted, were identified in anhydrobiotic embryos of *A. franciscana* (AfrLEA3m\_47, AfrLEA3m\_43, and AfrLEA3m\_29), and the corresponding mRNA species were isolated and the cDNA amplified (Boswell et al. 2014a). All three of these new mitochondrial LEA proteins (plus a fourth mitochondrial isoform previously reported, AfrLEA3m) are apparently products of independent genes based on mass spectrometry and sequence comparisons. AfrLEA2, predicted to be cytoplasmically localized based on bioinformatics (Hand et al. 2007), was shown to exist as a homodimer in *A. franciscana* (Boswell et al. 2014a).

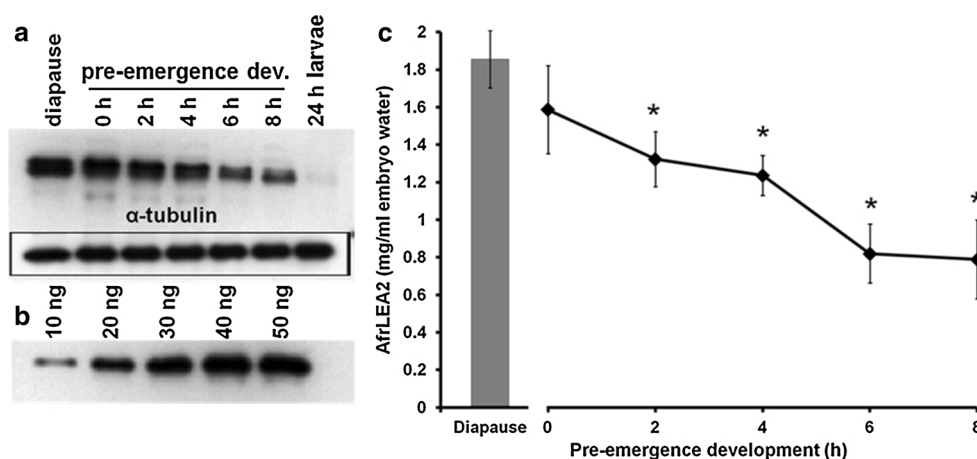
Quantification of protein expression for these proteins showed that cellular concentrations are highest in diapause embryos and decrease during development to low levels in desiccation-intolerant nauplius larvae (Fig. 1). The protein expression patterns are in agreement with the mRNA expression patterns previously reported for AfrLEA2 and AfrLEA3m (Hand et al. 2007; Menze et al. 2009). Few studies have attempted to rigorously estimate the effective cellular concentrations of LEA proteins. As a consequence, some functional roles projected from in vitro experiments may not be applicable in vivo because the concentrations used are often unrealistic. We found that the titer of the cytoplasmic-localized LEA protein (AfrLEA2) ranged from 0.8 to 1.8 mg/g cellular water across development, and the combined value for mitochondrial-targeted LEA proteins (AfrLEA3m, AfrLEA3m\_29, AfrLEA3m\_43) was 1.2–2.2 mg/ml matrix volume for post-diapause and diapause embryos, respectively. These units should be reasonably physiological considering the subcellular compartments in which the proteins reside (for methods of calculation, please see Boswell et al. 2014a). We find the work of Tolleter et al. (2007) on the mitochondrial-targeted LEAM in pea seeds to be particularly germane in this regard. When considering the matrix location of LEAM, these investigators calculated that in order to provide protection to about one third of the inner membrane surface (an estimate of the protein-free area), the protein theoretically must represent about 0.6 % of total matrix protein. With total matrix protein in the range of 400 mg/ml, the concentration needed would be 2.4 mg/ml LEAM, which is quite physiologically realistic considering our estimate that the three mitochondrial LEA proteins from *A. franciscana* embryos are present at a combined

concentration of 2.2 mg/ml. In summary, such estimates suggest that the effective concentrations of cytoplasmic vs. mitochondrial Group 3 LEA proteins are similar in vivo and provide guidance for the design of in vitro functional studies with these proteins.

### Structural and functional characterization of LEA proteins from *A. franciscana*

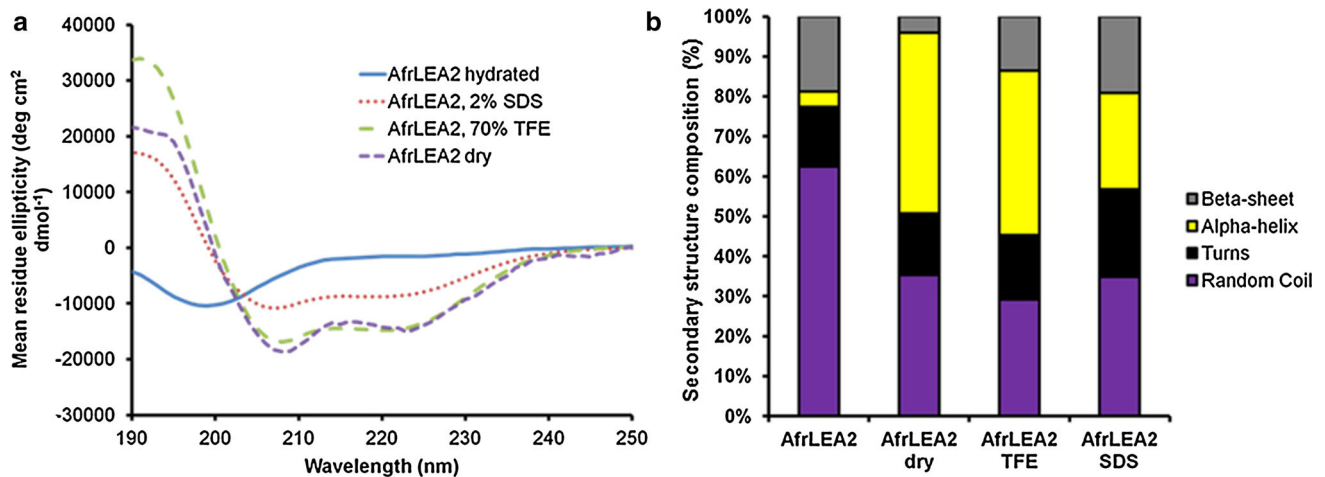
#### Protein structure is dependent on hydration state

Structural characterization is an important step in a comprehensive assessment of LEA proteins, and we recently completed investigations of the secondary structure of two Group 3 LEA proteins from embryos of *A. franciscana* (AfrLEA2 and AfrLEA3m) in aqueous solution, in solution with two different co-solvents, and in the dried state (Boswell et al. 2014b). Fully hydrated in solution, the CD spectrum of AfrLEA2 exhibited features typical of a disordered, random-coiled protein as judged by a 200-nm minimum of ellipticity (Fig. 2); application of appropriate algorithms underscored the low percentage of  $\alpha$ -helix content (Fig. 2). Desiccation of the protein caused an increase in  $\alpha$ -helix content from 4 % in solution to 46 % in the dried state. As previously shown for other LEA proteins in solution (e.g., Tolleter et al. 2007; Shih et al. 2012), addition of the desolvating agent trifluoroethanol (TFE) also promoted an increase in  $\alpha$ -helices, as did the addition of SDS. Similar results were found with AfrLEA3m, i.e., it was predominantly disordered in solution and adopted a more  $\alpha$ -helical structure after drying. AfrLEA3m possessed a greater percentage of  $\beta$ -sheet in the dry state compared to



**Fig. 1** Measurements of AfrLEA2 protein in extracts of *A. franciscana* by Western blot analysis. **a** Expression for AfrLEA2 is shown at various stages of the life cycle [diapause; pre-emergence development (hours 0, 2, 4, 6, and 8); and nauplius larvae (24 h)].  $\alpha$ -Tubulin was used as a loading control. **b** Concentration dependency of

recombinant AfrLEA2. **c** AfrLEA2 concentrations from diapause through 8 h of pre-emergence development. Asterisks indicate means are statistically different from diapause values (one-way ANOVA, Tukey,  $P < 0.05$ ). (After Boswell et al. 2014a)



**Fig. 2** Left panel CD spectra for recombinant AfrLEA2 in the hydrated state, in the presence of 2 % SDS, 70 % TFE, and after desiccation. Right panel secondary structure composition of

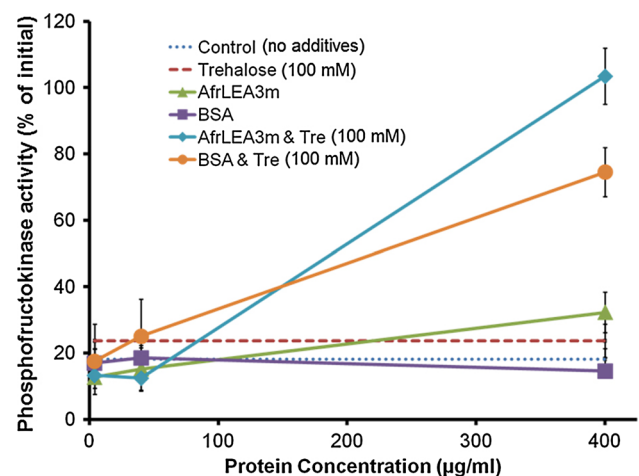
recombinant AfrLEA2 for the above conditions, as calculated from the respective CD data with appropriate algorithms (adapted from Boswell et al. 2014b)

AfrLEA2, which could explain the lower  $\alpha$ -helix content in AfrLEA3m. In the future it would be informative to evaluate the impact of trehalose on the structural features of these proteins from *A. franciscana* in the hydrated and dried states. Altering the speed of drying and assessing structures at intermediate states of water content could provide additional insights relevant to diverse drying conditions in nature. It could well be that the type of protection conferred by LEA proteins could differ as the severity of drying changes. For example, an individual LEA protein could function as a molecular shield in solution, and the same LEA protein could gain structure as water is removed to further protect the cell in the dry state by interacting with membranes, stabilizing sugar glasses, and forming filamentous networks (Tunnacliffe and Wise 2007; Hand et al. 2011b). In summary, our investigations into secondary structure revealed that these proteins, consistent with a number of LEA proteins from different sources, are intrinsically disordered in solution and gain defined structure during dehydration.

### Stabilization of target enzymes by LEA proteins varies depending on the target chosen

The ability of recombinant AfrLEA2 and AfrLEA3m, both alone and in concert with trehalose, to afford protection to different target enzymes during desiccation and subsequent rehydration was evaluated with the cytoplasmic enzymes phosphofructokinase (PFK) and lactate dehydrogenase (LDH) and the mitochondrial enzyme citrate synthase (CS). PFK is considered one of the most dehydration-sensitive enzymes known (Carpenter and Crowe 1988). After drying, PFK displayed a residual activity upon rehydration of 18 %, which was only increased to 24 % in the presence of

trehalose (Fig. 3). Our result with 100 mM trehalose alone was virtually identical to that reported by Carpenter et al. (1987) when those authors used a ‘slow-drying’ regime (20 h over a desiccant) very similar to the procedure that we used. However, when Carpenter et al. (1987) used a fast-drying regime, they found that trehalose stabilized PFK significantly better. Clearly the speed of drying impacts the efficacy of stabilization of PFK by trehalose. Gain of secondary structure by LEA proteins is also affected by speed of drying (Wolkers et al. 2001), with rapid drying promoting greater  $\alpha$ -helical structure. Addition of bovine serum albumin alone did not afford any protection to PFK during drying



**Fig. 3** Residual PFK activity after desiccation without additives (control) or in the presence of protectants. Values are reported as percent of the initial PFK activity measured prior to desiccation (mean  $\pm$  SD,  $n = 9$ ). AfrLEA3m plus trehalose provided a significantly higher level of protection than did any other treatment (one-way ANOVA plus Tukey,  $P \leq 0.05$ ) (redrawn from Boswell et al. 2014b)

(Fig. 3). However, the protection afforded by AfrLEA2 and AfrLEA3m was remarkable: 98 % of control (non-dried) activity was preserved when the enzyme was dried in the presence of AfrLEA2 plus trehalose (Li et al. 2012), and 103 % of control activity was preserved in the presence of AfrLEA3m plus trehalose (Fig. 3). To our knowledge this is the first time the protective ability of LEA proteins has been tested with a target protein possessing such high sensitivity to desiccation. The protective effects of the two LEA proteins with LDH as the target were very modest, whereas the protective abilities with CS as the target were intermediate between PFK and LDH. Thus the protection afforded to proteins during drying is dependent on the target chosen. Similar preferential protection has been reported for the interaction of a plant LEA protein with lipid membranes (Tolletier et al. 2010). However, based on this limited set of target proteins, it does not seem that a cytoplasmic-localized LEA protein protects cytoplasmic targets preferentially compared to a mitochondrial-resident target, nor that a mitochondrial-localized LEA protein does a superior job of stabilizing a mitochondrial target compared to the protection afforded by a cytoplasmic LEA protein.

### Subcellular localization of AfrLEA2 and AfrLEA3m in *A. franciscana* embryos

The subcellular distribution of these two LEA proteins has recently been verified in *A. franciscana* embryos (Boswell and Hand 2014) with immunohistochemistry and confocal microscopy, which was particularly challenging due to the amounts of yolk contained in these embryonic cells. Mitochondria in these cells do not form an extended reticulum, but rather are punctate in nature. Nevertheless, the results confirmed the mitochondrial location of AfrLEA3m (including subforms AfrLEA3m 47, AfrLEA3m 43 and AfrLEA3m 29) and the cytoplasmic/nuclear location of AfrLEA2 as predicted by bioinformatic analyses (Hand et al. 2007; Menze et al. 2009; Boswell et al. 2014a). The co-localization of AfrLEA3m with VDAC, indicated by the yellow color in merged images, supported the mitochondrial location (Fig. 4a–c). Co-localization of AfrLEA3m proteins with VDAC was not always uniform, which could be a consequence of the VDAC antibody having more consistent access to its antigen due to the location of VDAC in the outer mitochondrial membrane (Boswell and Hand 2014). However, immunoblotting of mitochondrial, cytoplasmic and nuclear fractions (Fig. 4d) confirmed the conclusion from confocal microscopy. Mitochondrial targeting of AfrLEA3m in embryos of *A. franciscana* is consistent with the localization of a chimeric protein composed of the AfrLEA3m leader sequence plus GFP to the mitochondrion in human HepG2 cells (Menze et al. 2009) and the

accumulation of AfrLEA3m in this organelle when transfected into HepG2 cells (Li et al. 2012; see below).

## Transfection of LEA proteins into cell lines

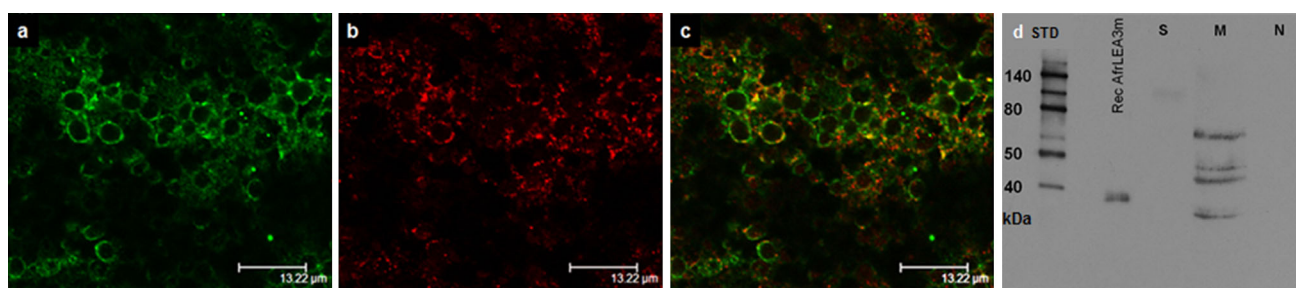
### Insect Kc167 cells

Two LEA proteins from Group 1 (AfLEA1.1 and AfLEA1.3) were cloned from embryos of *A. franciscana* and transgenically expressed in Kc167 cells derived from *Drosophila melanogaster* (Marunde et al. 2013) in order to test whether Group 1 proteins have the capacity to improve tolerance to water stress on desiccation-sensitive cells. These cells, as well as *D. melanogaster* as a species, do not endogenously express LEA proteins and do not display any appreciable tolerance to desiccation (cf. Gibbs and Matzkin 2001). Confocal microscopy revealed that a construct composed of green fluorescent protein (GFP) and AfLEA1.3 accumulated in the mitochondria, while AfLEA1.1-GFP was found in the cytoplasm. Cells expressing AfLEA1.3 showed significantly improved viability during hyperosmotic challenge in the presence of a non-permeant osmolyte (50–200 mM sucrose) and survived convective air drying to lower moisture contents compared to Kc167 controls (0.36 vs. 1.02 g H<sub>2</sub>O/g dry mass) (Fig. 5a). Acute titration of permeabilized cells with NaCl led to respiratory inhibition in Kc167 control cells that was ameliorated by 18 % in Kc167-AfLEA1.3 cells (Fig. 5b). Thus, AfLEA1.3 exerted a protective influence on mitochondrial function and viability of Kc167 cells during water stress. It is noteworthy that in these cases of moderate water and salt stress, AfLEA1.3 provided protection even under hydration states when the protein likely was still intrinsically disordered. Acquisition of  $\alpha$ -helical structure generally does not commence in LEA proteins until very low water contents—in the range of 20 % weight percent water and lower (Li and He 2009; Hand et al. 2011b). Consequently, it seems that the folded conformation of LEA proteins is not always required to confer protection against water stress or to exhibit functional attributes that are beneficial (e.g., Chakrabortee et al. 2007). In the study by Chakrabortee et al. (2007), a Group 3 LEA protein from a nematode reduced protein aggregation when co-expressed with self-aggregating polyglutamine proteins when cells were in a state of full hydration.

### Human HepG2 cells

AfrLEA2 and AfrLEA3m (Group 3 LEA proteins) were stably transfected into human HepG2 cells under control of a Tet-inducible gene expression system (Li et al. 2012), along with a constitutively expressed trehalose transporter





**Fig. 4** Subcellular localization of AfrLEA3m (and its closely related isoforms recognized by AfrLEA3m antibody) in embryos of *A. franciscana* visualized with confocal microscopy. Shown are the images for AfrLEA3m proteins (**a** green), VDAC (**b** red) and the merged image (**c** yellow) indicating areas of co-localization. **d** Immunoblot of AfrLEA3m for the supernatant (S), mitochondrial

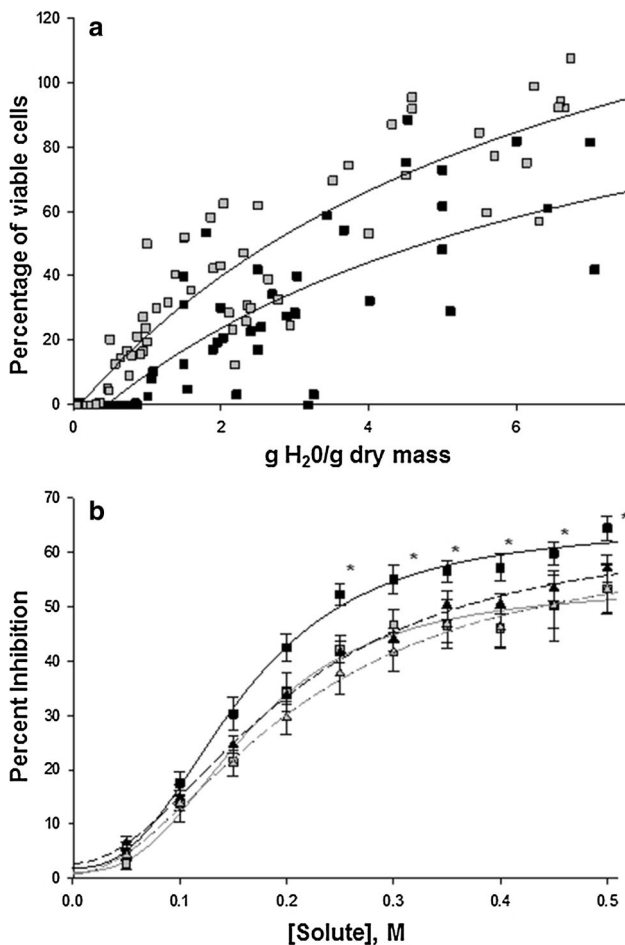
(M) and nuclear (N) fractions of extracts from *A. franciscana* embryos. The lane labeled ‘Rec AfrLEA3m’ was loaded with recombinant AfrLEA3m (deduced molecular mass of 34.1 kD that includes the mitochondrial targeting sequence) plus a 6X-His tag. The lane labeled ‘STD’ was loaded with the indicated markers for molecular mass (adapted from Boswell and Hand 2014)

for intracellular loading of this disaccharide (cf. Kikawada et al. 2007). A spin-drying technique was used to rapidly desiccate cells in 60 s to a water content of  $<0.12$  g H<sub>2</sub>O/g dry mass based on FTIR spectromicroscopy and bulk gravimetric analysis. Compared to traditional droplet drying, spin-drying avoids the slow increase in osmolarity that can damage cells due to salt stress, and the cracking and variable water loss kinetics that leads to samples that are not uniformly dried (Chakraborty et al. 2011a, b). Cells were removed from the spin-drying chamber and immediately rehydrated. Control cells without LEA proteins or trehalose exhibited 0 % membrane integrity, compared with 98 % in cells loaded with trehalose and expressing AfrLEA2 or AfrLEA3m (Fig. 6a). Even without intracellular trehalose, AfrLEA3m conferred 94 % protection based on membrane integrity. When these immediately rehydrated cells were placed in cell culture, proliferation across 7 days showed an 18-fold increase for cells dried with AfrLEA3m and intracellular trehalose, compared with 27-fold for non-dried controls (not statistically different; Fig. 6b). In contrast, the growth kinetics for cells expressing AfrLEA3m but without trehalose was markedly poorer than non-dried controls, which indicates the importance of intracellular trehalose in cell growth after drying/rehydration. Finally, growth kinetics for cells expressing AfrLEA2 (with or without trehalose) was significantly reduced compared to non-dried controls.

These results underscore the positive benefit of LEA proteins and trehalose for improving short-term stability of spin-dried cells. On the downside, however, viability is lost rapidly in minutes when dried cells were held at room temperature; loss of viability can be greatly retarded by immediate storage of the dried cells at liquid nitrogen temperatures (Chakraborty et al. 2011b). Lack of storage stability at room temperature represents a continuing challenge for biostabilization of dried cells. Multiple issues

likely contribute to the cellular instability. Were there sufficient quantities of glass-forming agents to promote cellular vitrification at room temperature?  $T_g$  for the spin-dried samples is approximately 4.5 °C, which indicates the lack of a vitrified state at room temperature. Stable, long-term storage of cells generally requires storage temperatures that are tens of degrees below the  $T_g$ . The estimated intracellular concentration of trehalose in these cells was ~20 mM. Increasing this concentration would elevate the  $T_g$  and predictably improve the storage outcomes at room temperature. Does trehalose penetrate key intracellular compartments (e.g., mitochondria, endoplasmic reticulum, Golgi)? The sugar distribution across subcellular compartments requires much more attention in desiccation studies, because it is well established that the benefit of trehalose is highest when present on both sides of biological membranes (Crowe et al. 2005), including the mitochondrial inner membrane (e.g., Liu et al. 2005). Are there detrimental changes in oxidative damage, redox status in the dried state under normoxia that prevent extended storage at ambient temperatures? Improvements in the long-term stability of spin-dried mammalian cells will require continued efforts on both the physicochemical and biological fronts.

For example, some Group 6 LEA proteins (termed seed maturation proteins, SMPs) may restore cellular functions after water stress by actually dissociating protein aggregates during rehydration (Boucher et al. 2010). In developing *Medicago truncatula* seeds, Group 6 LEA proteins preferentially accumulate in mature seeds, and importantly, one of these (D34.3) is strongly correlated with long-term viability of seeds in the dried state (Chatelain et al. 2012). The Group 6 LEA protein from *A. franciscana* (AfrLEA6; cf. Wu et al. 2011) that we have recently cloned, sequenced and expressed exhibits strong sequence homologies to SMPs in plants, including D34.3. AfrLEA6 is less hydrophilic than LEA proteins from Groups 1 and 3, and the



**Fig. 5 a** Viability of Kc167 cells from *Drosophila melanogaster* after drying and rehydration. Kc167 control cells (black) and cells expressing AfLEA1.3 (gray) were convectively dried in a solution containing 200 mM trehalose. Expression of AfLEA1.3 significantly improved the cellular response to desiccation (ANCOVA:  $F_1$ ,  $P < 0.0001$ ,  $r^2 = 0.74$ ). **b** Percent inhibition of respiration in permeabilized Kc167 control cells (square, solid line) and Kc167-AfLEA1.3 cells (triangle, dashed line) measured at various concentrations of NaCl (black) or KCl (gray, dashed line). Oxygen consumption was measured in presence of malate, glutamate, pyruvate, succinate and ADP ( $n = 4$ ,  $\pm$ SE). \*Significant differences between cell lines ( $P \leq 0.05$ ) (redrawn from Marunde et al. 2013)

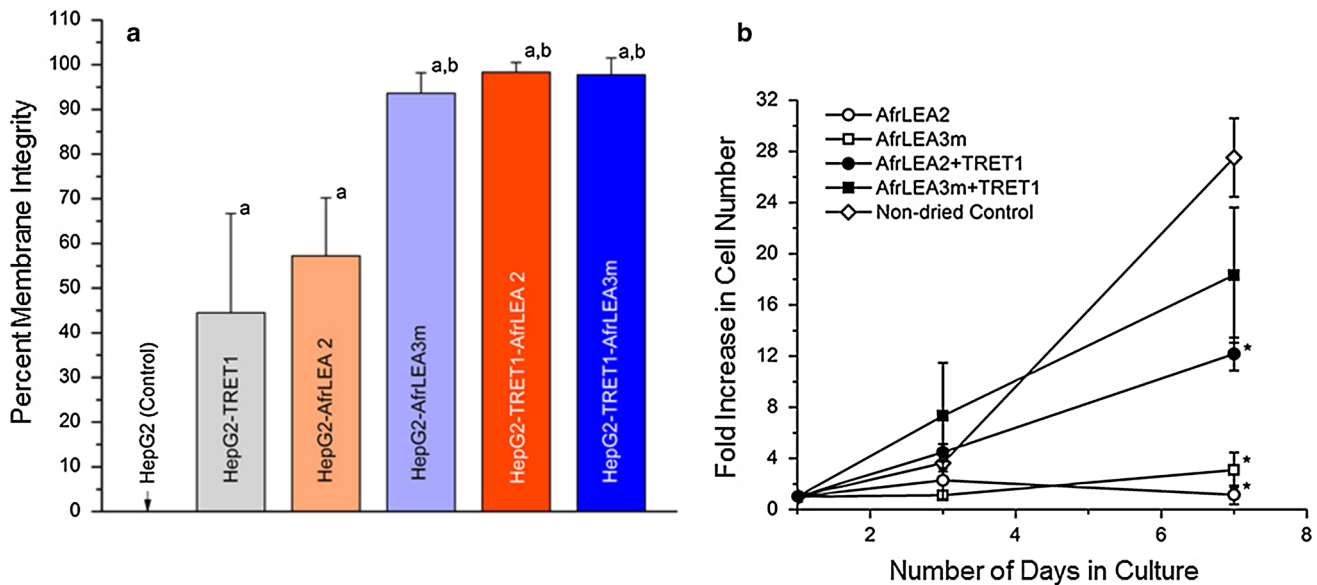
protein shares this characteristic with other SMPs like MtPM25 (Boucher et al. 2010). We are very interested in evaluating whether AfrLEA6 (Group 6) will function to improve long-term desiccation tolerance in animal cells as suggested for SPMs in plants (Chatelain et al. 2012).

### Metabolic preconditioning

Metabolic depression is typically correlated with extended survival during environmental stress, particularly for embryonic stages (Podrabsky and Hand 2015). Diapause as

seen in many invertebrates is a developmentally programed reduction of development and often metabolism, the depth of which can be profound. Entry into diapause and the associated metabolic depression is often observed in various organisms prior to exposure to environmental stresses in nature (Podrabsky and Hand 2015). For *A. franciscana* embryos, we have shown that a significant portion of the metabolic arrest was accomplished by restricting carbon substrate to the mitochondrion, which involved an orchestrated interplay at multiple enzymatic steps (Patil et al. 2013). For example, inhibition of pyruvate dehydrogenase entailed a time-dependent phosphorylation during diapause entry. In addition, metabolic arrest also involved diminished respiration through mitochondrial Complexes I and II and inhibition of the phosphorylation system (Patil, Gnaiger, Hand, pers. communication). These results provide key insights into mechanisms of metabolic downregulation.

Application of the concept of metabolic preconditioning, as illustrated by the diapause state, may extend survival during subsequent drying and water stress. As we have previously emphasized for diapause, one theme is that the AMP:ATP ratio is elevated and correlated with the arrest of metabolism (Hand et al. 2011a). A high AMP:ATP ratio stimulates by allostery and phosphorylation the AMP-activated protein kinase (AMPK), which is considered a metabolic fuel gauge for the cell (Hardie et al. 1998). Activation of AMPK inhibits protein synthesis and cell proliferation, which helps to conserve cellular energy in times of energy limitation (Hardie 2007; Ruderman et al. 2010). Accordingly, we tested the possibility that preconditioning cells by the activation of AMPK might improve cell survival during water stress. We used the membrane-permeant adenosine analogue AICAR (5-amino-4-imidazole carboxamide ribonucleoside), which is phosphorylated intracellularly by adenosine kinase to give ZMP (5-amino-4-imidazole carboxamide ribonucleoside monophosphate). This AMP analogue accumulates in cells and mimics the effects of AMP on AMPK. Application of AICAR prior to the freezing of various cell lines increased survivorship compared to the respective non-treated cells (Menze et al. 2010). Interestingly, the greater the depression of cell proliferation due AICAR, the greater is the survivorship post-freezing. Both the decreased cell proliferation and the increased survivorship across cell lines were correlated with the effective adenylate ratio modulated by AICAR:  $[(AMP) + (ZMP)]/(ATP)$ . Based on this positive result, additional types of metabolic preconditioning (cf. Borcar et al. 2013) are currently being tested to improve the biostabilization of cells during water stress.



**Fig. 6** Membrane integrity and long-term growth of HepG2 cells after spin-drying and immediate rehydration. **a** Control cells contained no intracellular trehalose or LEA proteins. HepG2-TRET1 cells contained approximately 20 mM trehalose; HepG2-AfrLEA2, expressed AfrLEA2 but without intracellular trehalose; HepG2-AfrLEA3m, expressed AfrLEA3m but without intracellular trehalose; HepG2-TRET1-AfrLEA2, expressed AfrLEA2 and contained intracellular trehalose; HepG2-TRET1-AfrLEA3m expressed AfrLEA3m and contained intracellular trehalose. All treatments were dried with extracellular trehalose present. Membrane integrity was determined using Syto-13 and ethidium bromide viability dyes. Values are mean  $\pm$  SD ( $n = 3$ –9 independent determinations). “a” indicates

statistically significant differences ( $P < 0.05$ ) vs. control (no trehalose or LEA protein), and “b” indicates statistically significant differences ( $P < 0.05$ ) vs. cells with intracellular trehalose alone. **b** Cells were immediately rehydrated and incubated in cell culture medium. The error bars indicate mean  $\pm$  SE. At day 7, cell numbers had increased by 18-fold for those with Afr-LEA3m plus trehalose, vs. 27-fold for non-dried controls (statistically equivalent;  $P = 0.21$ ). The growth kinetics for cells expressing AfrLEA2 (with or without trehalose) and Afr-LEA3m (without trehalose) were markedly lower compared with the non-dried controls ( $P \leq 0.05$ ), as indicated by asterisks (redrawn from Li et al. 2012)

## Concluding remarks

The above studies based on findings for the brine shrimp *A. franciscana* are helping to integrate our understanding of desiccation tolerance across multiple levels of biological organization from macromolecules to cells to organisms. Engineering desiccation tolerance in dehydration-sensitive cells and organisms is complex and undoubtedly will require multifactorial approaches beyond the transfection of cells with LEA proteins and the intracellular loading of small molecular protectants like trehalose. Nevertheless, initial transfection/loading studies of cells, coupled with metabolic preconditioning and innovative desiccation methods like spin-drying, are highlighting the strengths and deficiencies in current approaches to biostabilization in the dried state.

**Author contribution** Steven C. Hand and Michael A. Menze contributed to the generation and analysis of much of the data presented, and both participated in the writing of this review.

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