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RESEARCH ARTICLE

An insect antifreeze protein from *Anatolica polita* enhances the cryoprotection of *Xenopus laevis* eggs and embryos

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ABSTRACT

Cryoprotection is of interest in many fields of research, necessitating a greater understanding of different cryoprotective agents. Antifreeze proteins have been identified that have the ability to confer cryoprotection in certain organisms. Antifreeze proteins are an evolutionary adaptation that contributes to the freeze resistance of certain fish, insects, bacteria and plants. These proteins adsorb to an ice crystal's surface and restrict its growth within a certain temperature range. We investigated the ability of an antifreeze protein from the desert beetle *Anatolica polita*, *Ap*AFP752, to confer cryoprotection in the frog *Xenopus laevis*. *Xenopus laevis* eggs and embryos microinjected with *Ap*AFP752 exhibited reduced damage and increased survival after a freeze–thaw cycle in a concentration-dependent manner. We also demonstrate that *Ap*AFP752 localizes to the plasma membrane in eggs and embryonic blastomeres and is not toxic for early development. These studies show the potential of an insect antifreeze protein to confer cryoprotection in amphibian eggs and embryos.

KEY WORDS: Antifreeze protein, Cryoprotection, *Xenopus laevis*, Eggs, Embryos

INTRODUCTION

Effective cryoprotection is an important, unsolved, practical problem in medicine, pharmaceutical and food industries, and agriculture. Near-freezing and freezing temperatures are not ideal for the survival of eukaryotic organisms, but in nature there are a variety of compounds and strategies for freeze avoidance and freeze tolerance to enhance the survival of certain organisms that experience extremely low temperatures. Antifreeze proteins (AFPs) were originally discovered in the blood of Antarctic notothenioid fish (DeVries and Wohlschlag, 1969) and have since been identified in other fish (Chao et al., 1996b; Gronwald et al., 1998), insects (Clark and Worland, 2008), plants (Griffith and Yaish, 2004; Zhang et al., 2004) and bacteria (Garnham et al., 2008). AFPs are structurally diverse; however, all AFPs have thermal hysteresis properties and inhibit ice recrystallization (the growth of small ice crystals into large ones) via the direct binding of AFPs to the surface of ice crystals (Davies, 2014; Haridas and Naik, 2013). Thermal hysteresis is the ability of AFPs to depress the freezing point of the solution without significantly affecting the melting point (Bar Dolev et al., 2016; Devries, 1971). Insect AFPs are considered 'hyperactive' AFPs (Fuller, 2004; Scotter et al., 2006; Venketesh and Dayananda, 2008) relative to fish AFPs owing to their higher thermal hysteresis activity, a property that helps insects survive much colder temperatures on land (e.g. −40°C) than the temperatures fish encounter in the Arctic Ocean (e.g. −1 to −2°C) (Doucet et al., 2009). Although the exact mechanism of ice-growth inhibition at the molecular level has been much debated, it is likely that both the hydrophobic effect and hydrogen bonding contribute to AFP adsorption to ice (Garnham et al., 2011).

AFPs have been investigated as potential agents for cryoprotection because of their capacity to prevent ice crystal growth and ice recrystallization, a major cause of cell death during freeze–thaw. Cryoprotection of cells, tissues and embryos is one of the most promising areas of AFP applications, and AFPs have been shown to play a positive role in cell viability after freeze–thaw in tissues including blood (Chao et al., 1996a; Kim et al., 2015), sperm (Nishijima et al., 2014; Younis et al., 1998), oocytes (Arav et al., 1994; Lee et al., 2015a; Rubinsky et al., 1991; Wen et al., 2014), embryos (Baguisi et al., 1997; Li et al., 2020; Nishijima et al., 2014; Rubinsky et al., 1992), hearts (Amir et al., 2004), liver (Rubinsky et al., 1994) and other various biological samples (Brockbank et al., 2011; Falk et al., 2018; Halwani et al., 2014; Kawahara et al., 2009; Kratochvilova et al., 2017; 2019; Lee et al., 2015b; Tomás et al., 2019). However, other studies indicated that AFPs contributed no benefit or even reduced cell viability (Koushafar et al., 1997; Pham et al., 1999), which has been attributed to the needle-like ice crystals (Scotter et al., 2006) promoted by fish AFPs. Another potential application of AFPs is their ability to confer cold tolerance to genetically engineered transgenic plants (Duman and Wisniewski, 2014; Holmberg et al., 2001; Lin et al., 2011; Wang et al., 2008).

Another potential source of cell damage during the freeze–thaw process originates from membranes undergoing phase transition, which results in increased membrane permeability. Some subclasses of ice-binding proteins have been found to interact with membranes and were proposed to mitigate membrane leakage, yet only a few of them have been studied to date (Bar Dolev et al., 2016; Garner et al., 2008; Inglis et al., 2006; Kar et al., 2016; Rubinsky et al., 1990; Tomczak et al., 2002; Wang et al., 2008). In particular, both type I fish AFP and some antifreeze glycoproteins (AFGPs) have been shown to interact with model membranes (Garner et al., 2008; Tomczak et al., 2002). Type I AFPs partially insert into the membrane, whereas AFGPs are thought to localize peripherally to the membrane surface (Tomczak et al., 2002). The insect antifreeze protein *Mp*AFP149, which was cloned into transgenic tobacco, accumulates in the outer

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List of abbreviations

AFP	antifreeze protein
DSC	differential scanning calorimetry
GFP	green fluorescent protein
THA	thermal hysteresis activity
IRI	ice-recrystallization inhibition

layers of the cell wall based on fluorescence microscopy studies (Wang et al., 2008); however, no other studies have yet demonstrated insect AFPs interacting with membranes.

In the present study, we investigated the cryoprotective effects of an insect AFP on *Xenopus laevis* eggs and embryos. *Xenopus laevis* is a model organism that has provided fundamental insights into cell and developmental biology and is relatively easy to maintain in the laboratory environment. Benefits of working with *X. laevis* eggs and embryos are that they are large (i.e. 1–1.2 mm), easily manipulated by microinjection (e.g. with DNA, mRNA or protein) and provide an ample source of biochemically active cytoplasmic extracts. Frog embryos develop externally, which allows for experiments to be performed prior to and directly following fertilization. *Xenopus laevis* share a common evolutionary history with mammals and can serve as excellent model organism to provide insights into human conditions and diseases (Wheeler and Brändli, 2009). While *X. laevis* eggs and embryos are relatively easy to work with in the laboratory, there have not been any successful reports involving their cryoprotection. Studies have investigated intracellular and extracellular ice formation with *X. laevis* oocytes to gain insight into cryoprotection (Guenther et al., 2006; Kleinhans et al., 2006). In another study, ectopic expression of the aquaporin-3 channel in *X. laevis* oocytes resulted in plasma membrane permeability to glycerol, ethylene glycol and propylene glycol (Yamaji et al., 2006). Although these studies suggest that cryoprotection of *X. laevis* oocytes and eggs may be possible, effective approaches do not currently exist. Development of cryoprotection protocols would be of great interest to *Xenopus* investigators, allowing for long-term storage of oocytes and eggs (not currently possible) and facilitating studies with single clutches of oocytes and eggs, thereby eliminating batch-to-batch variability.

The present study deals with *Ap*AFP752, which originates from the desert beetle *Anatolica polita* found in the Gurbantunggut Desert within the Xinjiang province of China and parts of Central Asia (Liu et al., 2015; Mao et al., 2011a). *Ap*AFP752 is an ortholog of the antifreeze proteins DAFP from the beetle *Dendroides canadensis* (Amornwittawat et al., 2008; Jia and Davies, 2002) and *Tm*AFP from *Tenebrio molitor* (Graham et al., 1997), both of which are extensively disulfide bonded right-handed β-helical proteins (Daley et al., 2002; Liou et al., 2000), and the ice-binding β-sheet contain Thr-Cys-Thr motifs in which the Thr residues line up with the oxygen atoms of the ice crystals. It is important to note that most of the prior studies on AFP applications have utilized the better-characterized fish derived AFPs. AFPs from insects are thought to be more promising candidates for cryoprotection (Clark and Worland, 2008), because they are hyperactive and the ice morphology they promote is different from fish AFPs (i.e. 'lemon-shaped' versus 'needle-shaped' ice crystals, respectively) (Bar Dolev et al., 2016; Bar-Dolev et al., 2012). The protein is expressed in the laboratory as a thioredoxin-antifreeze fusion protein (Trx-*Ap*AFP752) and has been shown to protect *E. coli* cells against cold damage in a concentration-dependent fashion (Mao et al., 2011b) and to enhance the viability of human skin fibroblast cells after freeze–thaw (Falk et al., 2018; Kratochvilova et al., 2017; 2019). We now show that the insect *Ap*AFP752 may have the potential to be used as a cryoprotector in *X. laevis* eggs and embryos without being toxic to further development.

MATERIALS AND METHODS

Expression and purification of Trx-*Ap*AFP752

Expression and purification of Trx-*Ap*AFP752 was performed according to protocols we reported previously (Kratochvilova et al., 2017). In short, the recombinant plasmid pET32b containing the Trx-*Ap*AFP752 gene (Mao et al., 2011b) was transformed into *Escherichia coli* Rosetta-gami (DE3) competent cells (Novagen), and the protein was overexpressed using isopropanol-1-thio-β-D-galactopyranoside (IPTG). The cells were harvested using centrifugation, and the cells were lysed using a French press. The protein was purified via nickel-affinity chromatography and size-exclusion chromatography using a GE Healthcare ÄKTA purifier 900. Purity of the protein was assessed using SDS-PAGE and stained with Coomassie Blue. Once the sample was pure, the sample was buffer exchanged into a Tris buffer (20 mmol l^{-1} Tris, 20 mmol l^{-1} NaCl, pH 7.5), and the sample was concentrated to approximately 120 µmol l^{-1} protein concentration. A lyophilized protein sample was prepared in which the sample was lyophilized in buffer (50 mmol l^{-1} potassium phosphate, 20 mmol l^{-1} NaCl, 1 mmol l^{-1} NaN_3, pH 8.0) overnight (Kratochvilova et al., 2017) and stored at room temperature (~21°C) for 17 months. The lyophilized sample was rehydrated with 18.2 MΩ Milli-Q® water (EMD Millipore), and the sample was exchanged into the Tris buffer before cryoprotection studies. Heat inactivation of purified Trx-*Ap*AFP752 was achieved by incubating the protein at 60°C for 15–20 min.

Xenopus laevis egg and embryo microinjections

Xenopus laevis eggs and embryos were produced as previously described (Sive et al., 2000). Egg and embryo microinjections were performed using a Picospritzer III as previously described (Jevtic and Levy, 2015). To produce mRNA for microinjections, the AFP coding sequence was cloned from pET32b (a gift from Ji Ma, Xinjiang University) (Mao et al., 2011b) into pCS107-GFP-3STOP (a gift from John Wallingford, University of Texas at Austin) at EcoRI and XhoI to generate AFP pCS107-GFP-3STOP (pDL61). Subsequently, pDL61 was digested with EcoRI and NcoI to remove the green fluorescent protein (GFP). The overhangs were filled in with T4 DNA polymerase and the plasmid was ligated to circularize, generating AFP pCS107 (pDL62). Both pDL61 and pDL62 were linearized with KpnI, and mRNA was expressed and purified from the SP6 promoter using the mMessage mMachine kit (Ambion). Additional details about protein and mRNA microinjections are included in Tables S1 and S2. All *X. laevis* procedures and studies were conducted in compliance with the US Department of Health and Human Services Guide for the Care and Use of Laboratory Animals. Protocols were approved by the University of Wyoming Institutional Animal Care and Use Committee (assurance no. A-3216-01).

Freezing and imaging of *X. laevis* eggs and embryos

Details of how eggs and embryos were frozen and thawed are included in Tables S1 and S2. Embryo images were acquired with an Olympus SZX16 research fluorescence stereomicroscope, equipped with an Olympus DP72 camera, a 11.5× zoom microscope body and a SDFPLAPO1XPF objective. Whole-

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mount fluorescence immunocytochemistry of eggs was performed as previously described (Jevtic and Levy, 2015). The primary antibody was mouse anti-Trx (Genscript, A00180) used at 1:250, and the secondary antibody was anti-mouse Alexa Fluor 488 (Molecular Probes, A11001) used at 1:250. Isolation and imaging of embryonic blastomeres were performed as previously described (Jevtic and Levy, 2015). Western blots on embryo extracts and infrared quantification of band intensities were performed as previously described (Jevtic et al., 2015; Vukovic et al., 2016). The primary antibodies were rabbit anti-GFP (Invitrogen, A6455) and mouse anti-tubulin (Santa Cruz Biotechnology, sc-32293), both used at 1:1000. The secondary antibodies were anti-rabbit IRDye 800CW (Licor, 925-32211) and anti-mouse IRDye 680RD (Licor, 925-68070), both used at 1:20,000. Recombinant GFP-lamin B3 of known concentration was used to calculate the absolute levels of GFP-AFP expressed in embryos. Sample sizes were chosen based on previous experience and common practice in the field and are included in the figure legends. The effect size was not pre-specified as this is not common practice in cell biology. Sufficient numbers of biological replicates were performed for all experiments in order to draw statistically significant conclusions.

Thermal hysteresis activity by differential scanning calorimetry

Aqueous solutions of 180 µmol l^{-1} Trx-*Ap*AFP752 in buffer (50 mmol l^{-1} K_2HPO_4, 20 mmol l^{-1} NaCl, pH 8) were analyzed via differential scanning calorimetry (DSC, TA Instruments Q2000). Samples were first cooled to −40°C and held for 10 min so as to completely freeze the solution. The next cycle involved heating from −40 to 10°C at a rate of 1°C min^{-1} to completely melt the solution and to capture the total enthalpy of melt for the sample. Subsequently, a sequence of cycles were run that consisted first of a 1°C min^{-1} cooling back to −40°C followed by 1°C min^{-1} heating to a progressively higher hold temperature starting at −1°C and increasing by 0.1°C in the next heating cycle (Mao et al., 2011a). This cycling of heating and cooling profiles process continued through to a maximum of 0.5°C for hold temperature. In the initial heating cycles, the goal was to only melt a small fraction of the ice, hold the system at that hold temperature for 5 min to come to steady state, and then on cooling to capture the exothermic profile of the refreezing of that water fraction. In early cycles, the melting and freezing endotherms and exotherms maintained symmetry, where for one (or two) cycles the freezing temperature is observed (Fig. 1) at a temperature lower than the hold temperature (i.e. melting temperature for that cycle). This depression in freezing point is defined as the thermal hysteresis activity (THA). The derivative of that particular heat flow cycle also allows us to more easily observe the rapid change in slope at the onset of freezing near the first minimum just under 0°C (seen in Fig. S1). The data were processed using TA Universal Analysis software and plotted in Microsoft Excel.

Ice-recrystallization inhibition assays

Solutions of Trx-*Ap*AFP752 were prepared at 20, 10, 5.0, 2.5 and 1.0 µmol l^{-1} in 50 mmol l^{-1} K_2HPO_4, 20 mmol l^{-1} NaCl, pH 8 buffer. Approximately 15 µl of sample was loaded by capillary action into a 25 µl microcapillary tube (Drummond) and the ends were sealed in a flame (Tomczak et al., 2003). The tubes were positioned next to one another and secured with a small amount of aluminum tape on each end. The solutions were flash frozen over 10–15 s in a stream of N_2 gas coming from a liquid nitrogen tank, creating microcrystalline ice as seen in Fig. 2A. The microcapillary tubes were positioned in a home-built apparatus to hold the samples at −6.0°C while observing each sample under magnification for 20 h. An initial photograph at 0 h and end photograph at 20 h were compared to determine the extent of ice recrystallization across the different solutions. Images were captured using ProScope Microcapture Plus software.

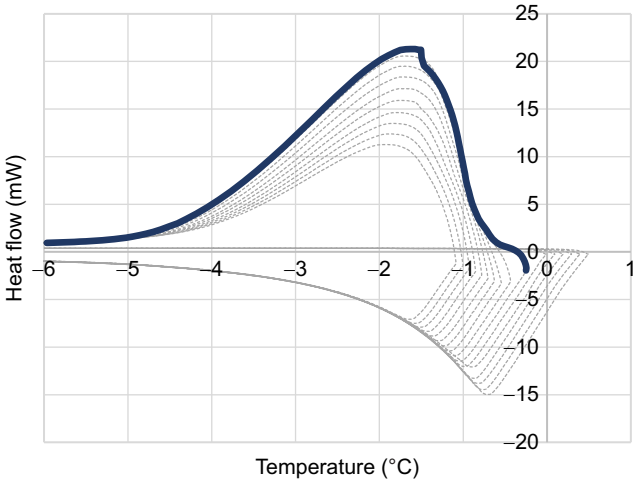

Fig. 1. Differential scanning calorimetry measurement of thermal hysteresis activity of 180 µmol l^{-1} Trx-*Ap*AFP752 (50 mmol l^{-1} K_2HPO_4, 20 mmol l^{-1} NaCl, pH 8). Negative heat flow endothermic profiles indicate partial melting of the ice, where positive exothermic profiles refer to the subsequent refreezing upon cooling. The solid exothermic profile refers to the particular cooling cycle used for thermal hysteresis activity analysis and shows the depression in the freezing point relative to the melting hold temperature for that cycle.

RESULTS

Antifreeze activity of AFP *in vitro*: thermal hysteresis and ice-recrystallization inhibition

To characterize the antifreeze activity of the recombinantly expressed and purified Trx-*Ap*AFP752, THA and the ice-recrystallization inhibition (IRI) activity assays were conducted. Differential scanning calorimetry (DSC) was used to assay THA. In Fig. 1, a series of heating and cooling cycles are shown for Trx-*Ap*AFP752 in buffer (50 mmol l^{-1} potassium phosphate, 20 mmol l^{-1} sodium chloride, pH 8). The sample was first cooled to −40°C so as to fully freeze the sample. Then, in each cycle, the sample was heated to a different subzero hold temperature and held isothermally for 5 min, which induced a partial melting of the sample. The melting endotherm associated with each heating cycle is shown as a dashed curve with negative heat flow. After the hold period, the respective cooling cycles follow with the exotherm on recrystallizing that fraction of water, seen as dashed profiles in the figure. The cycle where we can assess thermal hysteresis is emphasized with a solid profile in dark blue (where other cycles are shown in dashed gray) and the depression in the freezing point relative to the hold temperature is clearly observable. In this particular case, the hold temperature was −0.1°C and the freezing point was observed as a rapid change in slope of the cooling exotherm at −0.75°C; thus, we observe a THA of 0.65°C. Subsequent heating cycles (with higher hold temperatures) did not show an immediate freezing exotherm on cooling, but instead experienced supercooled freezing in the heterogeneous nucleation temperature range (observed at −22.9°C, −22.6°C and −21.6°C; see Fig. S1). We also wanted to assess whether lyophilized AFP

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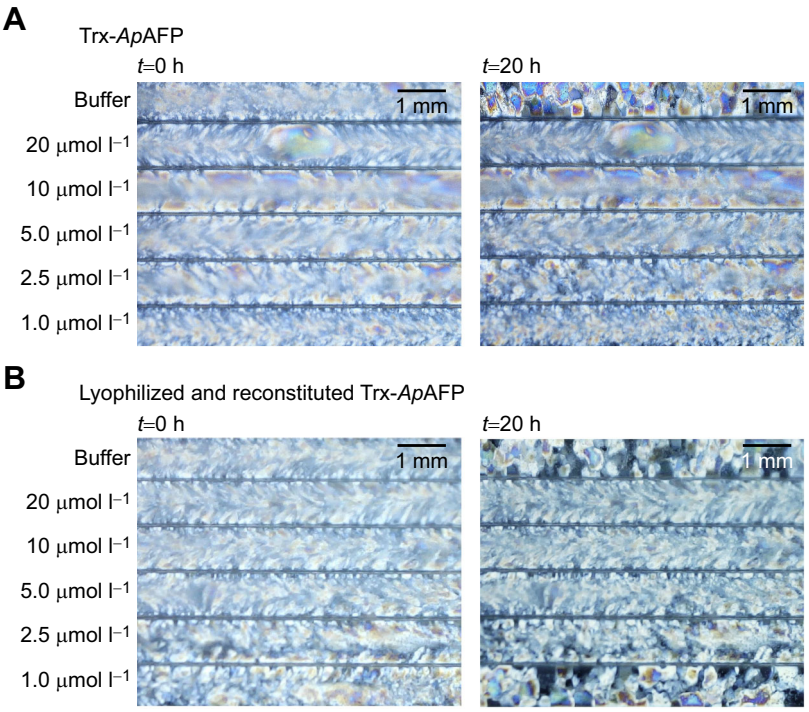

Fig. 2. Ice-recrystallization inhibition by Trx-*Ap*AFP752 at different concentrations before and after lyophilization. (A) Initial solutions, *t*=0 h, of buffer alongside varying concentrations of Trx-*Ap*AFP752 in microcapillary tubes after flash freezing to yield microcrystalline ice and the same solutions after holding for 20 h at −6.0°C, demonstrating significant ice recrystallization in the buffer but not the Trx-*Ap*AFP752 solutions. (B) A similar experiment using lyophilized and reconstituted Trx-*Ap*AFP752 that demonstrates significant ice recrystallization inhibition for all concentrations higher than 1.0 μmol l^{-1}. The solutions in these tubes were thawed and flash frozen to microcrystalline ice a second time, yielding the same ice recrystallization inhibition behavior of the Trx-*Ap*AFP solutions when followed over a 20 h period. The color saturation and brightness were adjusted of the lyophilized image at 20 h to better match the other three images.

retains cryoprotective activity, as lyophilization can be a very useful technique for protein storage. The AFP sample was lyophilized, reconstituted with water, and activity was reassessed. DSC showed retention of thermal hysteresis activity after lyophilization (Fig. S1).

IRI assays were performed before and after lyophilization. The IRI assays corroborate the THA performance and exhibit no change in ice-crystal granularity, indicating no change in the IRI activity at concentrations above 1 mmol l^{-1}. The IRI assays with Trx-*Ap*AFP752 demonstrate functional behavior at 5.0 μmol l^{-1} (Fig. 2), which is the concentration used for cryoprotection of the frog eggs. The concentration of Trx-*Ap*AFP752 necessary for ice recrystallization inhibition was consistent across batches of the protein. Both of these assay methods indicate that lyophilization does not functionally alter the activity of the AFP.

AFP confers some cryoprotective effects when microinjected into *X. laevis* eggs

We microinjected *X. laevis* eggs with purified recombinant AFP to a final concentration of ∼5 μmol l^{-1}. For control experiments, eggs were microinjected with an equal volume of buffer or AFP that had been heated. Microinjected eggs were submitted to a freeze–thaw cycle and imaged. The external morphology of eggs is commonly used to determine whether eggs are viable or dead, and it is well established that apoptotic eggs exhibit a white puffy appearance (Broadus et al., 2015; Du Pasquier et al., 2011; Gillespie et al., 2012; Iguchi et al., 2013; Johnson et al., 2010; Tokmakov et al., 2011; Willis et al., 2012), while lysed eggs break open and release their cytoplasmic contents. Therefore, we used the physical appearance of eggs to assess whether the eggs were apoptotic or lysed, which we refer to as 'damaged' for the rest of the study. Based on 12 independent experiments, 65±22% (mean±s.d.) of AFP-injected eggs were undamaged with normal appearance after freeze–thaw, as opposed to only 19±17% of control-injected eggs (Fig. 3, Table S1). This difference was highly statistically significant with a Student's *t*-test value of 6×10^{-6}, despite the variability between experiments that likely results from batch-to-batch differences in egg quality. In all experiments, AFP microinjection conferred a cryoprotective effect over the buffer or heat-inactivated controls. We tested a wide variety of different freezing conditions, including rapid freezing at −20°C for 15 min, slow freezing at −20°C overnight (by placing a dish of eggs in buffer into a surrounding iso-propanol bath), chilling on ice prior to freezing, and supplementing the surrounding buffer with 10% DMSO and 20% FBS. Freezing AFP-injected eggs in these various ways yielded roughly similar cryoprotective effects (Table S1). It is worth pointing out that supplementing the freezing medium with 5–10% DMSO did not confer any additive cryoprotective effect (data not shown). Taken together, these data strongly suggest that intracellular AFP protects eggs from damage caused by freezing and thawing.

As lyophilized AFP has maintained its THA and IRI activity *in vitro*, we wanted to assess whether lyophilized AFP also retains cryoprotective activity in eggs. Similar to what we observed with fresh AFP, 76±30% (*N*=4 experiments) of eggs injected with lyophilized and reconstituted AFP were undamaged versus only 30 ±17% of controls (Fig. 4A,B). AFP stored in solution at 4°C for 2 weeks retained roughly the same level of cryoprotective activity as fresh protein, while 3-week-old protein began to exhibit a loss of activity (Table S1). A sample of lyophilized AFP stored for over a year at room temperature retained full cryoprotective potential in our assay, thus lyophilization would appear to be the preferred method for long-term AFP storage. We also tested the effect of varying the concentration of microinjected AFP. Although ∼5 μmol l^{-1} AFP protected 65±33% (*N*=5 experiments) of microinjected eggs from damage, only 23±18% of eggs microinjected with ∼1–2 μmol l^{-1} AFP were undamaged, similar to controls, with 24±20% undamaged (Fig. 4C,D). We were unable to test AFP concentrations higher than 5 μmol l^{-1} because eggs microinjected with greater than 5% of the egg volume recover poorly and AFP stocks could not be stably concentrated further. Taken together, these data show that the cryoprotective effect of microinjected AFP is concentration-dependent, and that AFP cryoprotective activity is preserved after lyophilization and long-term storage. To begin to

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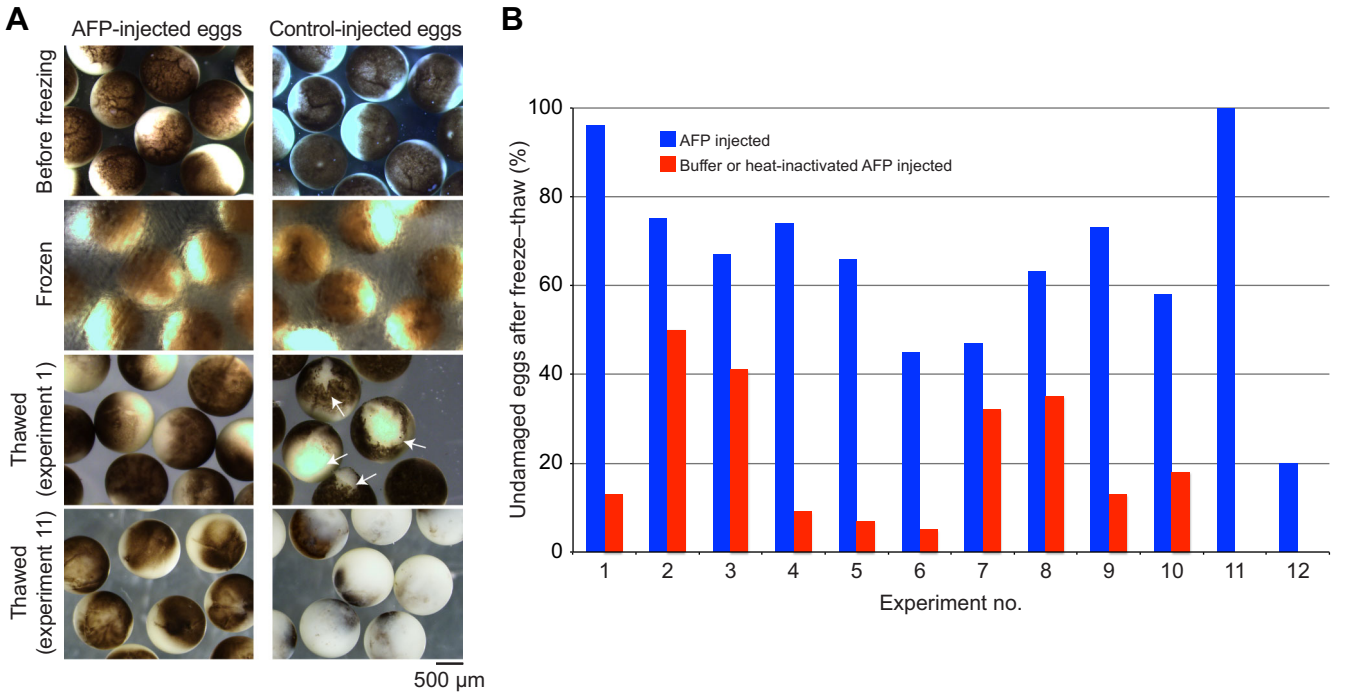

Fig. 3. Antifreeze protein (AFP) confers cryoprotection on *Xenopus laevis* eggs. (A) Eggs kept in 0.5× Ringer's+6% Ficoll were microinjected with 50 nl of 117 µmol l^{-1} Trx-*Ap*AFP752 (final AFP concentration within the egg ∼5 µmol l^{-1}, assuming an egg volume of ∼1 µl). For controls, eggs were microinjected with 50 nl of buffer (20 mmol l^{-1} Tris, 20 mmol l^{-1} NaCl, pH 7.5) or 50 nl of heat-inactivated AFP. After microinjection, eggs were incubated in the same buffer for 1 h at room temperature, moved to 5 ml 0.5× Ringer's without Ficoll, and placed at −20°C for 15 min until the buffer had frozen. Eggs were then thawed at room temperature for 45 min and imaged. In some experiments, egg damage was evidenced by rupture of the membrane, as in experiment 1 and indicated by white arrows. In other cases, damaged eggs turned completely white, indicative of apoptosis, as in experiment 11. (B) Twelve independent experiments were performed using different batches of eggs, and percentages of undamaged eggs were quantified. For experiments 11–12, all buffer-injected eggs were damaged. *n*=9–105 eggs per experiment and condition (average *n*=36). Table S1 summarizes egg numbers and conditions for microinjection and freezing for each experiment; in some experiments, overnight freezing was performed.

address the mechanism of action of AFP, we performed whole-mount immunocytochemistry on eggs to determine the localization of microinjected AFP. We determined that AFP preferentially localizes to the plasma membrane of eggs, perhaps in this way conferring a cryoprotective effect on the membrane (Fig. 5), which is important especially during phase transition.

Microinjected eggs cannot be fertilized

To assess whether microinjecting *X. laevis* eggs with AFP might represent a novel approach to embryo preservation, we attempted to fertilize eggs after a freeze–thaw cycle. We were unable to successfully fertilize AFP-injected eggs. To begin to understand the reason for this failure to fertilize, we performed a series of control experiments. We first tested how simply puncturing of eggs affected their sensitivity to freeze–thaw, finding that this physical manipulation of the egg resulted in roughly the same fraction of damaged eggs as control microinjections with buffer (Fig. S2A,B). Allowing longer times for the microinjection site to heal, from 45 min to 2 h, did not improve the percentage of undamaged eggs after freeze–thaw (data not shown). This result indicated that microinjection itself was damaging to eggs. We next subjected un-injected eggs to freeze–thaw and observed great variability in the fraction of undamaged eggs, ranging from 12% to 83% in six independent experiments (Fig. S2C). Generally, a greater fraction of AFP-injected eggs was undamaged compared with un-injected eggs (69±29% versus 39±25%), confirming the cryoprotective effect of the AFP (Fig. S2C). We also attempted freezing un-injected eggs in buffer containing 5 µmol l^{-1} AFP, but found that the presence of AFP in the surrounding buffer conferred no cryoprotective advantage (data not shown). These results indicate that freezing of un-injected eggs is not a reliable or robust approach for *X. laevis* cryoprotection.

We next wondered whether the failure to fertilize AFP-injected eggs might result from a general toxicity of the AFP. To test this, we fertilized eggs and then microinjected embryos with 5 µmol l^{-1} AFP. Without imposing a freeze–thaw, we allowed these embryos to develop and observed that AFP-injected embryos were capable of developing through gastrulation (Fig. S3, Table S3) and into tadpoles (data not shown), demonstrating that AFP is not generally toxic to the egg or embryo. Lastly, we tested whether the microinjection process itself caused eggs to become unfertilizable. Indeed, eggs that were punctured with a microinjection needle could not be fertilized, even those that were not subjected to a freeze–thaw cycle (data not shown), consistent with previous reports that pricking of eggs leads to their parthenogenetic activation (Wangh, 1989; Wolf, 1974). Taken together, although intracellular AFP clearly confers a cryoprotective effect on *X. laevis* eggs, administration of AFP through microinjection will likely not be a useful approach for *X. laevis* cryoprotection owing to microinjected eggs not being able to be fertilized.

Cryoprotection of AFP-injected *X. laevis* embryos is variable

Given that microinjected eggs cannot be fertilized, we wanted to test whether AFP-injected embryos might better survive a cycle of freezing and thawing. For these experiments, we first fertilized eggs and then microinjected 1-cell stage embryos with purified

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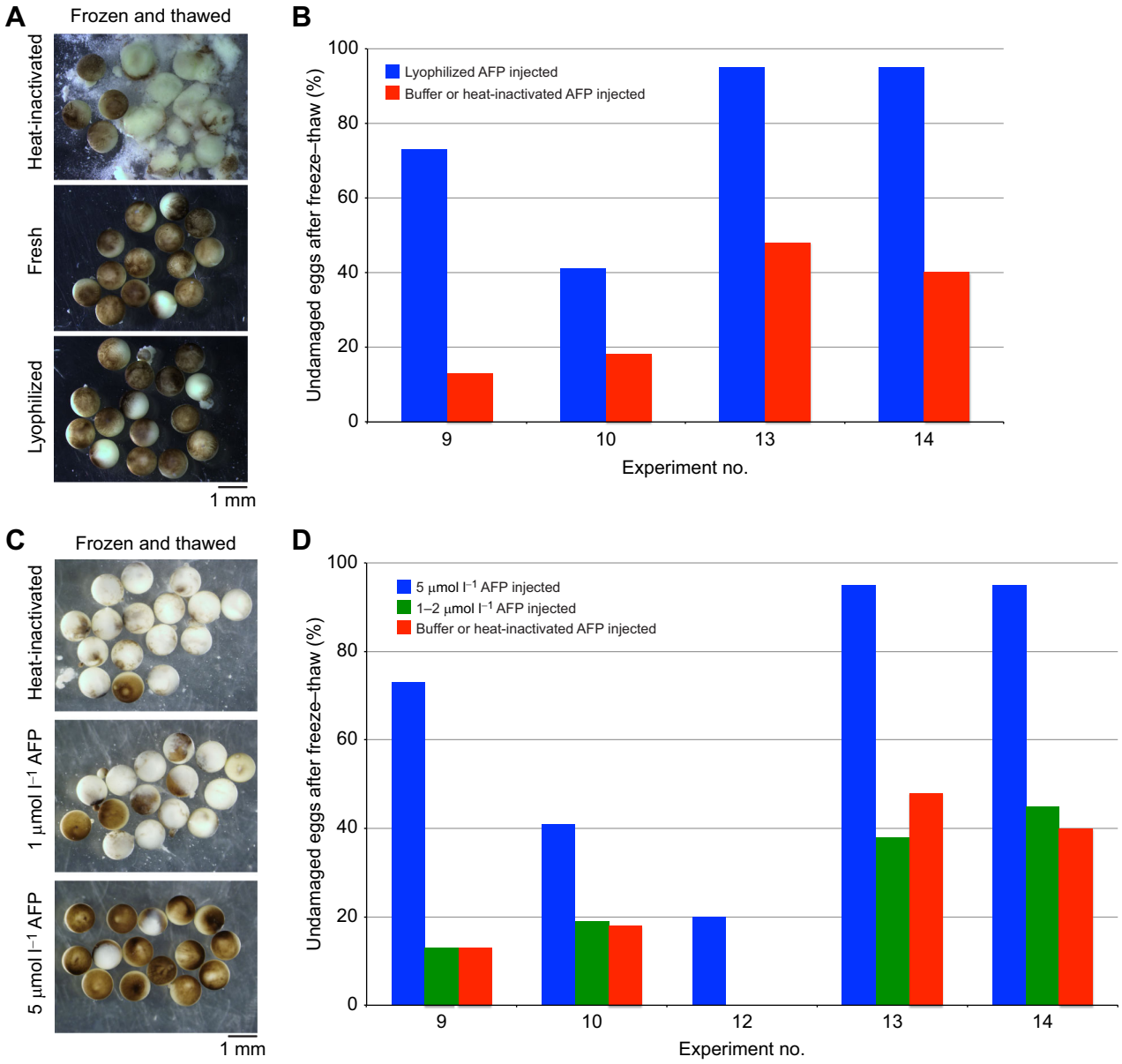

Fig. 4. Effects of AFP lyophilization and concentration on efficacy of *X. laevis* egg cryoprotection. (A) Eggs kept in 0.5× Ringer's+6% Ficoll were microinjected with 50 nl of either fresh AFP or AFP that had been lyophilized, stored at room temperature (21°C) for up to 17 months, and reconstituted. A final AFP concentration within the egg of 5 µmol l^{-1} was obtained in each case. For controls, eggs were microinjected with 50 nl of buffer (20 mmol l^{-1} Tris, 20 mmol l^{-1} NaCl, pH 7.5) or 50 nl of heat-inactivated AFP. After microinjection, eggs were incubated in the same buffer for 1 h at room temperature, moved to 5 ml 0.5× Ringer's without Ficoll, and placed at −20°C for 15 min until the buffer had frozen. Eggs were then thawed at room temperature for 45 min, imaged and scored for the effect of freeze–thaw. (B) Four independent experiments were performed using different batches of eggs, and percentages of undamaged eggs were quantified. *n*=15–40 eggs per experiment and condition (average *n*=34). Table S1 summarizes egg numbers and conditions for microinjection and freezing for each experiment (experiments 9, 10, 13, 14). (C) Eggs were microinjected with fresh AFP to obtain a final AFP concentration within the egg of either 5 or 1–2 µmol l^{-1}. For controls, eggs were microinjected with the buffer (20 mmol l^{-1} Tris, 20 mmol l^{-1} NaCl, pH 7.5) or heat-inactivated AFP. After microinjection, eggs were incubated in the same buffer for 1 h at room temperature, moved to 5 ml 0.5× Ringer's without Ficoll, and placed at −20°C for 15 min until the buffer had frozen. Eggs were then thawed at room temperature for 45 min, imaged and scored for the effect of freeze–thaw cycle. (D) Five independent experiments were performed using different batches of eggs, and percentages of undamaged eggs were quantified. For experiment 12, all eggs injected with buffer or 1 µmol l^{-1} AFP were damaged. *n*=13–40 eggs per experiment and condition (average *n*=30). Experimental conditions are summarized in Table S1 (experiments 9, 10, 12–14).

recombinant AFP to a final concentration of ∼5 µmol l^{-1}. For control experiments, embryos were microinjected with an equal volume of buffer or AFP that had been inactivated by heat treatment. Embryos were allowed to develop to the 4- or 8-cell stage (i.e. stage 4–5), submitted to a freeze–thaw cycle, and imaged to assess whether embryos were damaged. As with eggs, it is well established that blastomeres exhibiting a white and puffy appearance are apoptotic (Richard-Parpaillon et al., 2004; Shiokawa et al., 2008; Wroble et al., 2007). Based on seven independent experiments, 47 ±33% of AFP-injected embryos were undamaged after freeze–thaw, compared with 21±10% of control-injected embryos (Fig. 6, Table S2). In only four of seven experiments with embryos was cryoprotection by AFP observed, as opposed to 12 of 12 experiments with eggs. These results indicate that although AFP

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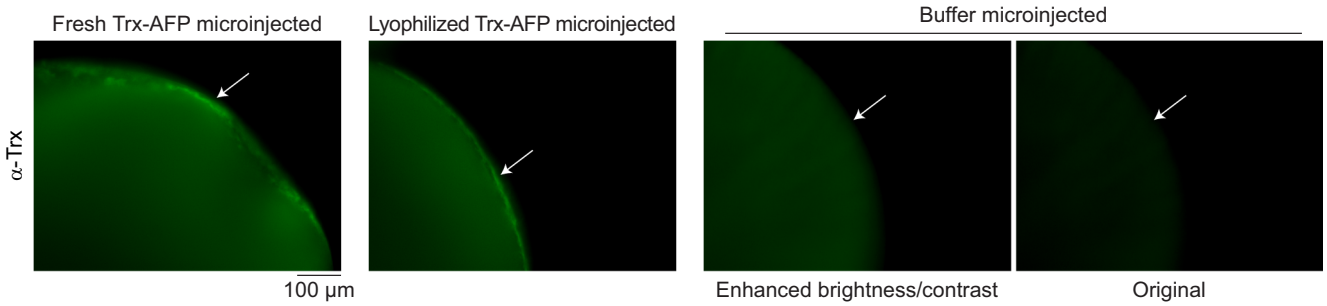

Fig. 5. AFP preferentially localizes to the plasma membrane of the egg. Eggs were microinjected with fresh or lyophilized/reconstituted Trx-*Ap*AFP752 to obtain a final AFP concentration within the egg of 5 µmol l^{-1}, or buffer as a control, as described in Fig. 3. After microinjection, eggs were incubated in the same buffer for 1 h at room temperature, fixed and processed for whole-mount immunocytochemistry using a primary antibody against Trx and Alexa Fluor 488 secondary antibody (green). The egg periphery is indicated with white arrows.

may confer some cryoprotection on embryos, the effect is variable and much less robust than that observed with eggs (Tables S1 and S2, Fig. 7). In a subset of experiments in which embryo cryoprotection was observed, we determined whether embryos were able to continue developing after freeze–thaw. Small numbers of AFP-injected embryos were able to develop to stage 7 or gastrula (Table S2), showing that some of these embryos retained viability after freeze–thaw; however, the inconsistency of the effect suggests that AFP microinjection of embryos is not a reliable method for *X. laevis* cryoprotection.

As an alternate approach to delivering AFP to embryos, we microinjected 1-cell stage embryos with mRNA encoding expression of AFP or GFP–AFP. Advantages of this approach are that the AFP is synthesized within *X. laevis* cells, as opposed to exogenously in bacteria, and it is possible to examine later stage embryos in which AFP expression is maintained. Embryos were allowed to develop to stage 8–9, allowing time for *in vivo* expression of the AFP, prior to freeze–thaw and imaging. As with the protein microinjections, the mRNA AFP microinjections produced variable effects, in some instances conferring cryoprotection and in other cases not (Fig. 8A,B). To assess the localization of AFP within live embryonic cells, we visualized GFP–AFP expressed from microinjected mRNA. The GFP–AFP was exclusively localized to the plasma membrane in embryonic blastomeres, similar to the localization of Trx–AFP in microinjected eggs, while GFP alone was entirely cytoplasmic (Fig. 8C). We also performed western blots on extracts from microinjected embryos and determined that the level of AFP expression from the microinjected mRNA ranged from 2.3 to 3.5 µmol l^{-1} (Fig. 8D). The variability of cryoprotection may be due to the low level (<5 µmol l^{-1}) of AFP expression. However, we did not attempt to measure the antifreeze activity (THA or IRI) of the AFP expressed in the frog embryos, and therefore we cannot verify that it had the same level of activity as the purified AFP protein that was microinjected into the eggs and embryos for the other experiments. Taken together, our data indicate that the ectopically expressed AFP was properly localized and non-toxic, yet induced variable cryoprotective effects in embryos. Thus, we propose that the greatest likelihood of

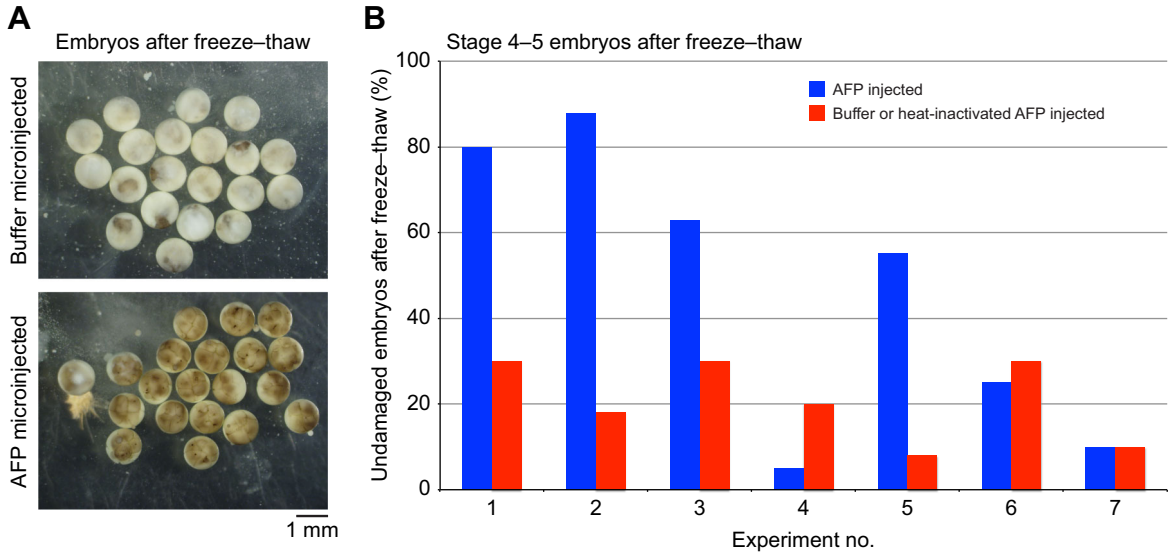

Fig. 6. The cryoprotective effect of AFP on *X. laevis* embryos is variable. (A) One-cell stage *X. laevis* embryos kept in 0.5× Ringer's+6% Ficoll were microinjected with 50 nl of 117 µmol l^{-1} Trx-*Ap*AFP. For controls, embryos were microinjected with 50 nl of buffer (20 mmol l^{-1} Tris, 20 mmol l^{-1} NaCl, pH 7.5) or 50 nl of heat-inactivated AFP. After microinjection, embryos were incubated in the same buffer at room temperature until they reached stage 4–5. Embryos were then moved to 5 ml 0.5× Ringer's without Ficoll and placed at −20°C for 15 min until the buffer had frozen. Embryos were then thawed at room temperature for 45 min and imaged. Damaged embryos turned white, indicative of apoptosis. (B) Seven independent experiments were performed using different batches of embryos, and percentages of undamaged embryos were quantified. *n*=10–40 embryos per experiment and condition (average *n*=22). Table S2 summarizes embryo numbers and conditions for microinjection and freezing for each experiment.

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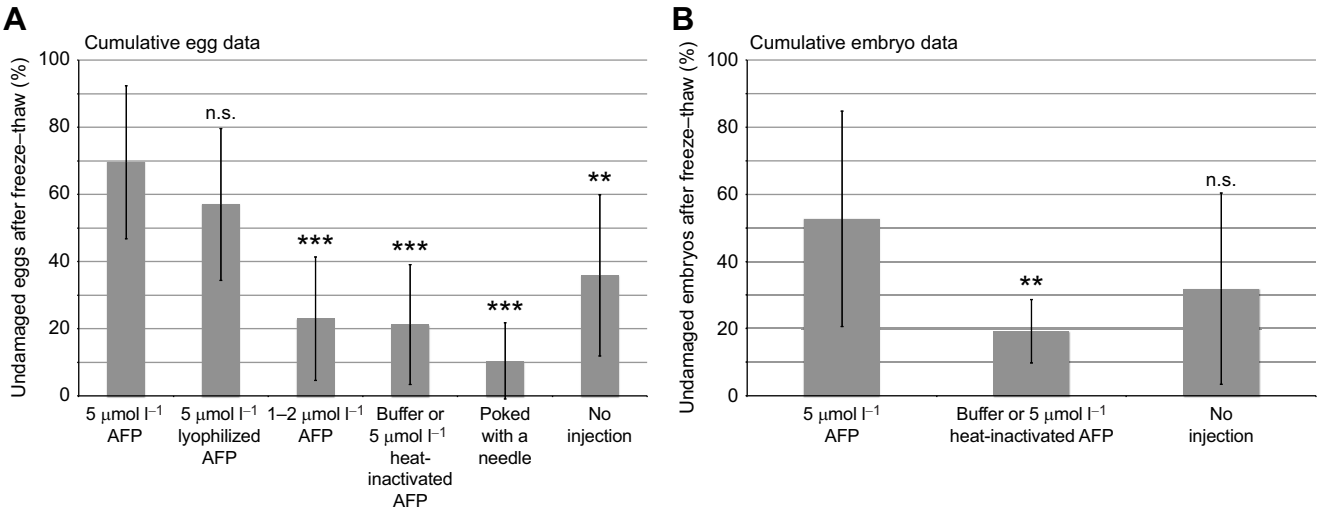

Fig. 7. Compiled microinjection data for eggs and embryos. (A) All data from egg microinjections shown in Table S1 were averaged and mean values are plotted. *n*=9–105 eggs per experiment and condition (average *n*=36). Two-tailed Student's *t*-tests were performed relative to the 5 µmol l^{-1} AFP microinjection data. (B) All data from embryo microinjections shown in Table S2 were averaged and mean values are plotted. *n*=10–40 embryos per experiment and condition (average *n*=20). Error bars are s.d. Two-tailed Student's *t*-tests were performed relative to the 5 µmol l^{-1} AFP microinjection data. ***P*<0.01; ****P*<0.001; n.s., not significant.

achieving cryoprotection in *X. laevis* will be to identify an approach to deliver AFP to eggs, rather than embryos, in a way that preserves the ability of those eggs to be fertilized after freezing and thawing.

DISCUSSION

Successful cryoprotection has important potential applications in the fields of medicine, industry and agriculture. In the last few decades, cryoprotection of human, cattle and mouse oocytes and embryos has been achieved using controlled-rate freezing and vitrification techniques. Cryoprotection of other vertebrate oocytes, eggs and embryos such as fish and amphibians is thought to be more difficult owing to their much larger volumes, lower surface-area-to-volume ratios and higher yolk contents. In particular, prior attempts have largely focused on vitrification of embryos from various fish species, with little success (Saragusty and Arav, 2011). Cryoprotection of cells, oocytes, embryos and organs from a few mammalian species at sub-zero temperatures using AFPs has been described (Brockbank et al., 2011), demonstrating the general feasibility of the approach. In the present study, we tested the cryoprotective effect of an antifreeze protein from the desert beetle *A. polita*, *Ap*AFP752, in *X. laevis* eggs and embryos.

We showed that intracellular AFP protects eggs from damage caused by freezing and thawing in a concentration-dependent manner, where 5 µmol l^{-1} AFP was more effective than 1–2 µmol l^{-1} AFP. An *in vitro* antifreeze activity assay confirmed that the purified recombinant AFP is a highly effective inhibitor of ice recrystallization at the low micromolar concentrations (at or over 1 µmol l^{-1}). In contrast, AFP THA assays indicated that the purified protein lowers the freezing point of water only moderately, by less than 1°C even at higher concentration (180 µmol l^{-1}); thus IRI activity is likely a much more important contributor to cryoprotection in AFP-injected *X. laevis* eggs. We have shown that the AFP cryoprotective activity is preserved after lyophilization and long-term storage both *in vitro* and *in vivo*, which is advantageous for potential applications of the AFP. Interestingly, the distribution of the injected AFP is not even or random in *X. laevis* eggs; AFP localizes to the plasma membrane preferentially, and the cells will experience a concentration gradient of AFP. Membrane stabilization by the AFP likely contributed to its cryoprotective activity, a mechanism that has already been shown in some ice-binding proteins (Bar Dolev et al., 2016; Garner et al., 2008; Inglis et al., 2006; Kar et al., 2016; Rubinsky et al., 1990; Tomczak et al., 2002; Wang et al., 2008). AFP-injected embryos develop normally into tadpoles, demonstrating that AFP has no toxic effect on developing embryos. When embryos were microinjected with AFP or mRNA encoding AFP, the cryoprotective effect was variable and much less robust than that observed with eggs (Tables S1 and S2, Fig. 7). This might be due to differences in the plasma membrane composition of eggs and early embryos, given that the process of fertilization induces massive changes in the egg's membrane.

Our results show that intracellular AFP has the greatest and most reproducible cryoprotective effects in eggs; however, administering AFP through microinjection renders those eggs unfertilizable. Fertilization of eggs involves calcium release from the endoplasmic reticulum, and this rise in cytoplasmic calcium levels leads to depolarization of the egg membrane and exocytosis of cortical granules, thus ensuring against polyspermic fertilizations (Kline, 1988; Whitaker, 2006). All of these events are induced by the physical manipulation of poking (e.g. microinjecting) the egg, explaining why the egg cannot undergo normal fertilization after microinjection. In the future, we propose to undertake an alternate approach for intracellular delivery of AFP to the egg. Cell-penetrating peptides are short peptide sequences that translocate freely through cell membranes. A type III AFP was delivered using Pep-1 into cells in a 2-D mammalian cell monolayer system by Tomás et al. (2019). Another cell-penetrating peptide, TATp-D, has been shown to have an enhanced ability to penetrate the cell membrane at sub-micromolar concentrations without causing cell toxicity (Futaki, 2006; Inomata et al., 2009; Monreal et al., 2015; Tomás et al., 2019). Pep-1 or TATp-D are excellent potential egg delivery agents for *Ap*AFP752 that might allow for improved cryoprotection of *X. laevis* eggs while maintaining their competency for fertilization.

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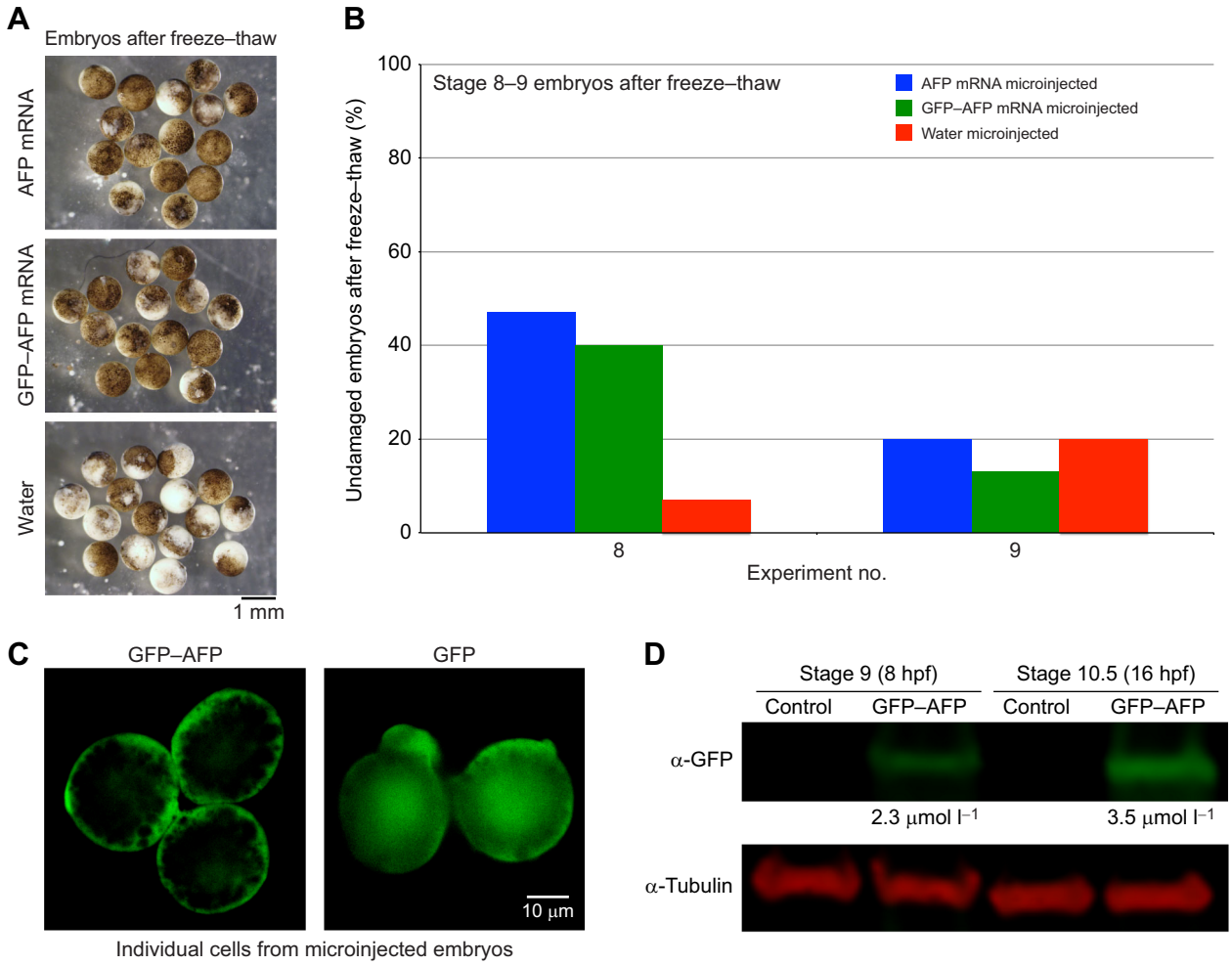

Fig. 8. AFP expressed from microinjected mRNA in *X. laevis* embryos localizes exclusively to the plasma membrane but confers variable cryoprotection. (A) One-cell stage embryos kept in 0.5× Ringer's+6% Ficoll were microinjected with 500 pg of mRNA encoding AFP or GFP–AFP, or 10 nl of water as a control. After microinjection, embryos were incubated at room temperature until they reached stage 8-9, allowing time for expression of the AFP. Embryos were placed in 5 ml 0.5× Ringer's without Ficoll at −20°C for 15 min until the buffer had frozen. Embryos were then thawed at room temperature for 45 min and imaged. Damaged embryos turned white, indicative of apoptosis. (B) Two independent experiments were performed using different batches of embryos, and percentages of undamaged embryos were quantified. *n*=15 embryos per experiment and condition. Table S2 summarizes embryo numbers and conditions for microinjection and freezing for each experiment (experiments 8 and 9). (C) One-cell stage embryos were microinjected with 500 pg of mRNA encoding GFP-AFP or GFP only. Embryos were incubated at room temperature until they reached stage 8–9, and live isolated blastomeres were imaged. (D) One-cell stage embryos were microinjected with 500 pg of mRNA encoding GFP–AFP or water as a control. Embryos were incubated at room temperature until they reached stage 9 or 10.5. To measure the level of GFP–AFP expression in embryos, western blots were performed on embryo extracts, probed for GFP or tubulin (for normalization), and quantified by infrared fluorescence. The concentrations noted below the GFP–AFP lanes indicate the calculated concentrations of GFP–AFP expressed in embryos at the indicated stages of development, determined by comparing band intensities for GFP–AFP and a GFP-tagged protein of known concentration run on the same gel.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: D.L.L., K.V.; Methodology: D.L.L., K.V.; Validation: D.L.L., K.V.; Formal analysis: P.J., P.W.B., J.G.T., D.L.L., K.V.; Resources: D.L.L., K.V.; Data curation: P.J., K.W.E., S.E.W., J.A.S., K.J., I.B.L., P.W.B., J.G.T., D.L.L., K.V.; Writing - original draft: P.J., K.W.E., S.E.W., P.W.B., J.G.T., D.L.L., K.V.; Writing - review & editing: P.J., P.W.B., J.G.T., D.L.L., K.V.; Visualization: J.G.T., D.L.L., K.V.; Supervision: P.W.B., D.L.L., K.V.; Project administration: D.L.L., K.V.; Funding acquisition: D.L.L., K.V.

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