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Citation: Voulgaridou, Georgia-Persephoni, Tsochantaridis, Ilias, Mantso, Theodora, Franco, Rodrigo, Panagiotidis, Mihalis and Pappa, Aglaia (2017) Human Aldehyde Dehydrogenase 3A1 (ALDH3A1) Exhibits Chaperone-Like Function. The International Journal of Biochemistry & Cell Biology, 89. pp. 16-24. ISSN 1357-2725

Published by: Elsevier

URL: <https://doi.org/10.1016/j.biocel.2017.05.017>
<<https://doi.org/10.1016/j.biocel.2017.05.017>>

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Accepted Manuscript

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PII: S1357-2725(17)30107-3
DOI: <http://dx.doi.org/doi:10.1016/j.biocel.2017.05.017>
Reference: BC 5133

To appear in: *The International Journal of Biochemistry & Cell Biology*

Received date: 15-1-2017
Revised date: 18-4-2017
Accepted date: 8-5-2017

Please cite this article as: Voulgaridou, Georgia-Persephoni., Tsochantaridis, Ilias., Mantso, Theodora., Franco, Rodrigo., Panayiotidis, Mihalis I., & Pappa, Aglaia., Human Aldehyde Dehydrogenase 3A1 (ALDH3A1) Exhibits Chaperone-Like Function. *International Journal of Biochemistry and Cell Biology* <http://dx.doi.org/10.1016/j.biocel.2017.05.017>

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Human Aldehyde Dehydrogenase 3A1 (ALDH3A1) Exhibits Chaperone-Like Function

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Abstract

Aldehyde dehydrogenase 3A1 (ALDH3A1) is a metabolic enzyme that catalyzes the oxidation of various aldehydes. Certain types of epithelial tissues in mammals, especially those continually exposed to environmental stress (e.g., corneal epithelium), express ALDH3A1 at high levels and its abundance in such tissues is perceived to help to maintain cellular homeostasis under conditions of oxidative stress. Metabolic as well as non-metabolic roles for ALDH3A1 have been associated with its mediated resistance to cellular oxidative stress. In this study, we provide evidence that ALDH3A1 exhibits molecular chaperone-like activity further supporting its multifunctional role. Specifically, we expressed and purified the human ALDH3A1 in *E. coli* and used the recombinant protein to investigate its *in vitro* ability to protect *SmaI* and citrate synthase (from precipitation and/or deactivation) under thermal stress conditions. Our results indicate that recombinant ALDH3A1 exhibits significant chaperone function *in vitro*. Furthermore, over-expression of the fused histidine-tagged ALDH3A1 confers host *E. coli* cells with enhanced resistance to thermal shock, while ALDH3A1 over-expression in the human corneal cell line HCE-2 was sufficient for protecting them from the cytotoxic effects of both hydrogen peroxide and tert-butyl hydroperoxide. These results further support the chaperone-like function of human ALDH3A1. Taken together, ALDH3A1, in addition to its primary metabolic role in fundamental cellular detoxification processes, appears to play an essential role in protecting cellular proteins against aggregation under stress conditions.

KeyWords: Aldehyde dehydrogenase 3A1; ; , corneal crystallin, molecular chaperone

1. Introduction

Molecular chaperones are proteins that facilitate other newly synthesized or denaturated proteins to stabilize and/or to fold to their native, functional structures (Hartl et al., 2011; Hartl and Hayer-Hartl, 2009). Proper protein function is a key element of cellular homeostasis and crucially important to cells for coping with environmental or intrinsic stress (Morimoto and Cuervo, 2014). It is known that oxidative stress is a common cause of cellular proteotoxicity since oxidation of proteins results in their structural disruption and consequently in the formation of inactive, aggregation-prone intermediates (Mirzaei and Regnier, 2006; Niforou et al., 2014; Trougakos et al., 2013).

Cells have developed strategies aiming to enhance both the efficiency of proteins with chaperone activity as well as their overall antioxidant capacity to maintain proteostasis under oxidative stress conditions (Miyata et al., 2012). For instance, 2-Cys peroxiredoxin is an abundantly expressed, antioxidant enzyme that catalyzes the reduction of various peroxide substrates, thus controlling cellular peroxide levels. Under redox stress conditions, though, 2-Cys peroxiredoxin displays holdase-like chaperone activity, which is triggered by the oxidation of highly sensitive cysteine residues of the protein. Oxidation of these cysteines induces a conformational switch of 2-Cys peroxiredoxin which allows it to form high molecular weight complexes with misfolded proteins and thus prevent their aggregation (Jang et al., 2004; Kumsta and Jakob, 2009; Perkins et al., 2015). Similarly, alpha-crystallin (α -crystallin) is a small heat-shock protein responsible for the optical properties of the lens. Its abundant expression facilitates short-range organization in the cytoplasm of the lens fibers, thus diminishing the scattering of light (Jester, 2008), while its holdase chaperone activity prevents lens opacification, by inhibiting the aggregation of aged and/or denaturated proteins (Posner et al., 2012).

Aldehyde dehydrogenase 3A1 (ALDH3A1) is a NAD(P)⁺-dependent enzyme that oxidizes medium chain aldehydes to their corresponding carboxylic acids (Pappa et al., 2003b; Pappa et al., 2001; Piatigorsky, 2000). It has recently been characterized as a corneal crystallin being constitutively expressed at high levels in the mammalian cornea (Jester et al., 1999; Pappa et al., 2005; Pappa et al., 2003a; Voulgaridou et al., 2016). ALDH3A1 appears to exert a variety of antioxidant and cytoprotective properties in corneal epithelial cells and prevents the development of opacifications in the cornea under oxidative stress conditions thus suggesting a potential mechanism acting as a chaperone-like molecule, analogous to α -crystallin (Black et al., 2012; Lassen et al., 2007; Pappa et al., 2005; Pappa et al., 2003a; Pappa et al., 2003b; Stagos et al., 2010). Indeed, a number of findings like: (i) ALDH3A1 up-regulation after eyelids openings in mice, (ii) its down-regulation in rat corneal keratocytes cultured under dark conditions (Jester, 2008) and (iii) the increased formation of opacities in the cortex and corneal haze phenotype in *Aldh3a1* (-/-) knock-out mice compared to wild-type mice (Lassen et al., 2007; Chen et al., 2016), support the notion that ALDH3A1 has a significant role in the maintenance of corneal optical properties. However, if such a role is accomplished through a chaperone-like activity remains elusive.

In this study, we were prompted to investigate the potential holdase chaperone activity of human ALDH3A1 and explore its protective role under stress conditions.

2. Material and methods

2.1 Materials

Expression vector pMAL-c2x was obtained from New England Biolabs (Beverly, MA, USA) and pet26b(+) vector by Novagen (EMD Millipore Corporation, Billerica, MA, USA). Primers for the cloning were purchased by Invitrogen (Carlsbad,

CA, USA), Pfu polymerase and DNA ligase kit from Fermentas (Burlington, ON, Canada) while the restriction enzymes and the chaperone plasmid set were from Takara (Shiga, Japan). Ni-NTA resin was from Qiagen (Venlo, Netherlands), and amylose resin was purchased from New England Biolabs (Beverly, MA, USA). Transformer TM kit for site-directed mutagenesis was obtained from Clontech (Takara). The antibiotics, the medium for the bacterial cultures, as well as the inducers, were obtained either from Applichem (Darmstadt, Germany) or Sigma-Aldrich Co. (Taufkirchen, Germany). Citrate synthase from porcine heart, oxaloacetic acid, acetyl-CoA along with protease inhibitors and all chemicals for the ALDH3A1 enzymatic activity were obtained from Sigma-Aldrich Co (Taufkirchen, Germany). Human corneal epithelium HCE-2 cell line was obtained from ATCC (Manassas, VA, USA) while the culture media along with the various additives, fetal bovine serum (FBS), antibiotics and trypsin were either from Biosera (East Sussex, UK), Gibco (Life Technologies, Carlsbad, CA, USA), Sigma-Aldrich Co. (Taufkirchen, Germany) or Biochrome (Berlin, Germany). Lipofectamine were from Life Technologies (Carlsbad, CA, USA). Hygromycin was from Carl Roth GmbH (Karlsruhe, Germany). The oxidants used in the study were either obtained from Sigma-Aldrich Co. (Primers, dNTPs, Trizol and Platinum SYBR Green were purchased from Invitrogen (Life Technologies Carlsbad, CA, USA) while the random hexamers and PrimeScript Reverse Transcriptase were from Takara (Shiga, Japan).

2.2 Expression and purification of recombinant human ALDH3A1

Expression and purification of recombinant ALDH3A1 fused with the maltose-binding protein (MBP) or a hexapeptide of histidines (6his) were performed as previously described (Voulgaridou et al., 2013).

2.3 *Sma*I restriction enzyme activity assay

The assay was performed as described previously with minor modifications (Santhoshkumar and Sharma, 2001). One unit of the restriction enzyme *SmaI* was heat-inactivated (90 min, 37°C) in the presence or absence of increasing concentrations of recombinant human ALDH3A1 in a final volume of 14 µl 1x *SmaI* buffer. After heat inactivation, 1 µl (200 ng) of plasmid DNA was added, and the reaction mixture was further incubated at 30°C for 90 min. *SmaI* activity was monitored by visualization of the cleaved product of plasmid DNA after subjecting the digestions to agarose gel electrophoresis and ethidium bromide staining.

2.4 Citrate synthase thermal aggregation assay

Thermal aggregation of citrate synthase assay was conducted as described previously (Santhoshkumar and Sharma, 2001) with a few minor alterations. Citrate synthase from porcine heart (75 µg) diluted in 1 ml of 40 nM HEPES (pH7.5) was incubated at 43°C in the presence of various concentrations (0-500 nM) of recombinant human ALDH3A1, while its aggregation was measured spectrophotometrically at 340nm (Libra S22 spectrophotometer) as an increase in optical density, due to light scattering.

2.5 Citrate synthase inactivation assay

The assay was performed as described earlier (Buchner et al., 1998; Santhoshkumar and Sharma, 2001). Specifically, a mixture of 100 ng citrate synthase and 70nM of recombinant ALDH3A1 was diluted in 930 µl TE buffer (50 mM Tris, 2mM EDTA, pH 8.0) and incubated at 37°C for 70 min. The remaining activity of the citrate synthase was then determined at several time points after the addition of 10 µl oxaloacetic acid, 10 µl 5-5'-dithio-bis(2-nitrobenzoic acid) (10 mM) and 30 µl acetyl-CoA (5 mM) in TE buffer by monitoring the increase in absorbance at 412 nm on a Libra S22 spectrophotometer.

2.6 Site-directed mutagenesis

Site-directed mutagenesis was performed to introduce a specific base change altering the codon for catalytic cysteine 243 to serine (Allali-Hassani and Weiner, 2001; Khanna et al., 2011) on the hALDH3A1 plasmid construct (Voulgaridou et al., 2016) through the Transformer TM kit by Contac according to the manufacturer's instructions. Briefly, two oligonucleotides were synthesized, a selection primer, intended to deactivate the unique restriction site for *NruI* (underlined sequence) in hALDH3A1 plasmid construct and a mutagenic primer, designed to incorporate the desired base change in the coding region of ALDH3A1 cDNA:

1. Selection primer: 5'- C AGC CTC GCG GCG CGC ACG CCA GCA AG - 3' and,
2. Mutagenic primer: 5'- GC CAG ACC TCC GTG GCC CCT G -3'.

The two primers were simultaneously annealed to one strand of the denatured hALDH3A1 plasmid DNA, and the mutated strand was further synthesized and ligated using T4 DNA polymerase and T4 DNA ligase.

Digestion with the *NruI* restriction enzyme ensured the selection of the constructs with the mutated *NruI* restriction site. The mixture of the digested plasmids was then transformed into mutS *E.coli*, a strain with defective mismatch repair mechanism, in order to be amplified and isolated. Subsequently, the mixed plasmid pool was digested once more with the *NruI* restriction enzyme and re-transformed in *mutS E.coli* cells. Single colonies were selected and their plasmid DNA was isolated and analyzed sequencing to confirm the Cys 243 mutation (hALDH3A1^{mut}).

2.7 pET-26b(+)/ALDH3A1^{mut} vector construction

The cloning of ALDH3A1^{mut} cDNA to the expression vector pet-26b(+) (pET-26b(+)/hALDH3A1^{mut}) was performed similarly to the construction of the pET-

26b(+)/hALDH3A1 plasmid (Voulgaridou et al., 2013). The mutated form of ALDH3A1 was amplified from the plasmid hALDH3A1^{mut} through PCR reaction with a pair of oligonucleotides designed to introduce an *NdeI* restriction site at the 5' end (restriction site underlined) and an *XhoI* restriction site at the 3' end (restriction site underlined) of the amplicon:

1. Forward primer: 5'-GGGAATTCCATATGAGCAAGATCAGCGAG-3' and,
2. Reverse primer: 5'-CCGCTCGAGGTGCTGGGTCAT-3'.

The PCR conditions were: 95°C for 90 sec (hot start), 95°C for 30 sec (denaturation), 56°C for 90 sec (annealing) and 72°C for 120 sec (extension) for 30 cycles and a final extension step at 72°C incubation for 10 min. The *NdeI*/*XhoI* fragment of the PCR reaction was ligated into the *NdeI* and *XhoI* restriction sites of the pET-26b(+) expression vector. Finally, the pET-26b(+)/hALDH3A1^{mut} construct was verified by double digestion with the *NdeI* and *XhoI* restriction enzymes, as well as, sequence analysis of the insert from both ends.

2.8 Dot heat shock assay

Dot heat shock assay was conducted using a previously described protocol with few modifications (Liu et al., 2009). Specifically, 10 ml of fresh LB were inoculated with 100 µl of an overnight culture of *E.coli*BL21(DE3) cells (transformed with pet26b(+), pet26b(+)/ALDH3A1 or pET-26b(+)/hALDH3A1^{mut}). Cultures were then cultivated at 37°C under shaking until they reached an OD₆₀₀ value of ~0.5 when IPTG (0.5mM) was added, and protein expression was performed at 25°C for 2 hours. Cultures were then fixed to 4x10⁸ of cells/ml and were serially diluted, while 3 µl of each dilution were spotted onto LB agar plates, subjected to thermal shock at 65°C for 1 hour and allowed to recover overnight at 37°C.

2.9 Thermotolerance assay

Thermotolerance assay was performed as described earlier with minor alterations (Takeuchi, 2006). Specifically, an overnight culture of *E.coli* BL21(DE3) cells (transformed with pet26b(+), pet26b+)/ALDH3A1 or pET-26b(+)/hALDH3A1^{mut}) was used for the inoculation of 10 ml of fresh LB. The culture was incubated at 37°C until it reached a value of OD₆₀₀~0.5. Then, protein induction was initiated by adding 0.5 mM of IPTG. Following 2 hours of incubation at 25°C, cells were diluted to 4x10⁷ cells/ml and were incubated at 42°C (3 h), 47.5°C (1h), or 52°C (1h). Subsequently, cultures were serially diluted, and 100 µl of each were plated on LB agar plates and incubated overnight at 37°C. LB agar plates were scanned, and single colonies were counted with Image J software.

2.10 Cell culture

Human corneal epithelium cell line HCE-2 were maintained in 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F-12 nutrient mixture (DMEM/F12) supplemented with 15% FBS, 0.5% (v/v) dimethyl sulfoxide (DMSO), 0.1 µg/ml cholera toxin, 10 ng/ml epidermal growth factor, 5 µg/ml insulin, 40 µg/ml gentamycin, 100 µg/ml streptomycin and 100 units/ml penicillin. HCE-2 stably transfected cell line was cultured in the same medium with the extra addition of 0.2 mg/ml hygromycin. Cells were cultivated at 37°C with 5% CO₂ in a humidified incubator.

2.11 Establishment of stably transfected HCE-2 cell line constitutively expressing human ALDH3A1

HCE-2 cell line was transfected with the full-length human ALDH3A1 subcloned into a suitable mammalian expression vector as previously described (Voulgaridou et al., 2016). HCE-2 cells (2x10⁶) were transfected with 16 µg of DNA (vector alone or

ALDH3A1/vector) with Lipofectamine reagent 2000. Stably transfected clones (mock/HCE-2 ALDH3A1/HCE-2) were selected under the pressure of hygromycin (0.2 mg/ml in culture medium). Finally, the expression of ALDH3A1 in the selected clones was assayed by real time PCR, enzymatic activity assay and Western blot analysis as described previously (Voulgaridou et al., 2016).

2.12 Sulforhodamine B (SRB) assay

For SRB assay, 10×10^3 of HCE-2/ALDH3A1 or HCE-2/mock cells were seeded in 96-well culture plates and were treated 24-h post plating, in triplicates, with tert-butyl hydroperoxide or H_2O_2 . Both oxidants were prepared in PBS as stock solutions of 50mM and subsequently diluted in cell culture medium into working concentrations of 0-200 μ M or 0-1000 μ M for tert-butyl hydroperoxide and H_2O_2 respectively. Following a 72-h incubation, the cell viabilities and the EC_{50} values of the oxidants for HCE-2/ALDH3A1 and HCE-2/mock cells were estimated as previously described (Voulgaridou et al., 2016).

2.13 Statistical analysis

At least three independent experiments were performed for each sample per condition tested. GraphPad Prism software (version 5) was used for all statistical analyses. Student's *t*-test was applied for comparison of results between two groups, and Dunnett post hock test was performed for assessing differences between individual groups. All values are expressed as the mean \pm S.E A value of $p < 0.05$ was considered significant.

3. Results

3.1 ALDH3A1 protects *SmaI* from thermal inactivation

A time-course thermal inactivation experiment for *SmaI* at 37°C and 42°C determined the experimental conditions to be used subsequently. Samples containing 1 unit of

enzyme were pre-incubated at 37°C and 42°C for 30, 60 and 90 min respectively. Then, 200 ng of plasmid DNA were added in the reactions which were further incubated at 30°C for 90 min. Residual *SmaI* activity in the heated samples was assayed by monitoring its ability to digest plasmid DNA (Figure 1A). The time-course experiments indicated that *SmaI* was inactivated after 90 min at 37°C and 60 min at 42°C incubation time. We chose the more moderate stress incubation conditions at 37°C for 90 min for the subsequent *SmaI* thermal deactivation experiments.

Then, we investigated the potential protective effect of various concentrations of the recombinant ALDH3A1 protein (MBP- or 6xHis-tagged ALDH3A1) against *SmaI* deactivation at 37°C for 90 min. Our results indicated that both the MBP- and 6xHis-tagged ALDH3A1 were able to protect *SmaI* from thermal inactivation at concentrations ≥ 53.3 nM (Figure 1B, 1C respectively). In order to exclude the possibility that the MBP could contribute to the protective effect of the ALDH3A1/MBP protein, MBP alone was also tested for its ability to protect *SmaI* under the same conditions. Results showed that no such protection was demonstrated thus indicating that the observed protection of the ALDH3A1/MBP fused protein appears to be ALDH3A1-specific (Figure 2A). To verify the reliability of the assay, we also examined BSA, lysozyme, and denatured recombinant ALDH3A1 (boiled for 10 min) for their potential to protect *SmaI* under the same conditions. BSA is known for its ability to stabilize and protect other proteins from thermal and chemical inactivation, whereas no such protective effect is displayed by lysozyme. For this reason, they are considered ideal control proteins for molecular chaperone assays (Hess and FitzGerald, 1998; Morrow et al., 2006; Olszewski and Wasserman, 1986; Santhoshkumar and Sharma, 2001). As expected, BSA was capable of maintaining *SmaI* activity at concentrations ≥ 533 nM while lysozyme exhibited no such protective effect (Figure

2B). Furthermore, denatured 6xHis-tagged ALDH3A1 did not demonstrate any protective effect on the target protein indicating that the chaperone-like function strongly depends on the native status of ALDH3A1 (Figure 2B).

3.2 ALDH3A1 prevents thermal aggregation and inactivation of citrate synthase

Citrate synthase (CS) is a thermally unstable protein that makes it a suitable model protein for studying and analyzing chaperone activities. Here, CS was incubated at 43°C, and its aggregation was monitored by the increase in absorbance at 360 nm. A protective effect was evident with both MBP- and 6xHis-tagged ALDH3A1 recombinant proteins in a dose-dependent manner (Figure 3A and 3B respectively). CS is known to get readily inactivated at 37°C. Therefore, we examined whether the presence of ALDH3A1 could also contribute to the maintenance of CS enzymatic activity during incubation at 37°C. Within the first minutes of incubation, CS started losing activity, and by 60 min, most of its activity was lost (Figure 4). Quite interestingly, while the MBP-tagged ALDH3A1 protein did not show significant protection against thermal deactivation of CS (Figure 4A), the 6xHis-tagged ALDH3A1 contributed significantly in maintaining CS enzymatic activity (Figure 4B). Moreover, CS retained approximately 70% of its initial activity even after 70 min of incubation at 37°C in the presence of ALDH3A1 (Figure 4B). Overall, the protection on CS heat inactivation displayed by 6xHis-tagged ALDH3A1 is in accordance with the restriction enzyme experiments described earlier.

3.3 ALDH3A1 exhibits a protection effect under thermal stress conditions

Thus far, our results indicate that the recombinant human ALDH3A1 was efficient in protecting the examined proteins from aggregation and/or inactivation under stress conditions *in vitro*. However, to assess if ALDH3A1 also functions as a chaperone-like

molecule *in vivo* we designed a series of experiments based on our previous observations (recombinant 6xHis-tagged ALDH3A1 was found to have increased specific enzymatic activity compared to MBP-tagged ALDH3A1 (Voulgaridou et al., 2013) in combination with current results (showing that 6xHis-tagged ALDH3A1 possesses superior chaperone-like potential) in order to assess if the enzymatic activity could be associated with the protective effects of the protein. Our results indicated that transformed *E. coli* cells expressing human wild-type or catalytically inactive ALDH3A1 remain viable and retain their ability to form colonies, following heat shock treatment. Dot heat shock assays revealed that expression of the 6xHis-tagged wild-type ALDH3A1 increased significantly the survival of *E. coli* cells against thermal stress induced at 65°C for 1 hour (Figure 5A). Interestingly, a strong protective effect was also observed when the catalytically mutant ALDH3A1 protein (ALDH3A1/6xHis^{mut}) was expressed in *E. coli* cells suggesting that the integrity of the active site is not essential for the protective effect of the protein (Figure 5A). The 6xHis-tagged recombinant ALDH3A1 expression was associated with increased survival colony numbers in thermotolerance assays performed at 42°C (Figure 5B.i), 47°C (Figure 5B.ii), and 52°C (Figure 5B.iii) for 1 hour.

3.4 ALDH3A1 exhibits a protection effect under oxidant stress conditions

To investigate the potential protective effect of ALDH3A1 under stress conditions in human corneal epithelial cells, we stably transfected the human corneal epithelial cell line HCE-2 with a suitable mammalian expression cloned with the cDNA of human ALDH3A1 or with the empty expression vector (mock transfected). The expression of ALDH3A1 was validated in the isolated transfected clones by Western blot analysis. The clone which demonstrated the highest protein levels (Figure 6A) was designated as ALDH3A1/HCE-2 and was selected for all the subsequent experiments.

The mRNA levels of ALDH3A1 in the ALDH3A1/HCE-2 cells were found to be >160-fold higher compared to mock/HCE-2 cells by real time PCR (comparative CT method) (Figure 6B). The enzymatic activity of ALDH3A1 in the ALDH3A1/HCE-2 cells was estimated to be 597 ± 43 units/min.mg, whereas mock/HCE-2 cell line exhibited negligible enzymatic activity (Figure 6C). ALDH3A1 expression was frequently monitored to confirm the maintenance of its stable expression.

Next, we examined the effect of various oxidants on the viability of the isogenic cell line pair. ALDH3A1/HCE-2 and mock/HCE-2 cells were incubated with increasing concentrations of tert-butyl hydroperoxide (Figure 7A) and hydrogen peroxide (H_2O_2) (Figure 7B) for 72 hours, and SRB assay was utilized to estimate cell viability. ALDH3A1 expression was associated with increased tolerance to tert-butyl hydroperoxide and H_2O_2 cytotoxicity, as indicated by the shifted to the right cell viability curves of the ALDH3A1-expressing cells compared to the mock transfected HCE-2 under both conditions tested. In the case of tert-butyl hydroperoxide, the EC_{50} values of ALDH3A1/HCE-2 and mock/HCE-2 cells were $38.11 \pm 1.37 \mu M$ and $24.71 \pm 4.7 \mu M$ respectively, resulting in 1.5-fold resistance of ALDH3A1/HCE-2 cells to tert-Butyl hydroperoxide compared to mock/HCE-2 cells (Figure 7A). In the case of H_2O_2 also, ALDH3A1/HCE-2 cells exhibited approximately a 2-fold resistance compared to the mock/HCE-2 cells (Figure 7B).

4. Discussion

ALDH3A1 is a cytoprotective corneal crystallin with multiple antioxidant modes of action and with an alleged role in the maintenance of corneal optical properties. To investigate the potential chaperone activity of ALDH3A1, we performed assays commonly used for studying proteins with holdase activity (Buchner et al., 1998;

Buchner et al., 1991; Carver et al., 2002; Hess and FitzGerald, 1998; Horwitz, 1992; Kumar et al., 2005a; Kumar et al., 2005b; Olszewski and Wasserman, 1986; Raman et al., 1995; Reddy et al., 2002; Santhoshkumar and Sharma, 2001). Our results indicated that both MBP and 6xHis fusion ALDH3A1 recombinant proteins were able to maintain the enzymatic activity of *SmaI* as well as the solubility of CS under thermal stress conditions. On the contrary, only 6xHis-tagged ALDH3A1 prevented the heat-induced inactivation of CS. Additionally, dot heat shock and thermotolerance assays revealed that the expression of both wild-type and mutant 6xHis-tagged ALDH3A1 resulted in higher colony formation efficiency and enhanced tolerance to thermal stress in BL21(DE3) *E. coli* cells. The cyto-protective role of ALDH3A1 was also evident under oxidative stress conditions in the human corneal epithelial cell line HCE-2.

Our results add to previously published data on the contribution of ALDH3A1 to the optical properties of the cornea. Specifically, the study by Ness et al. (2002) was among the first to examine whether ALDH3A1 serves as a structural component in the cornea and similarly to lens crystallins. Their experiments established that ALDH3A1 is not mandatory for the obtainment of corneal transparency, as *Aldh3a1* (-/-) knockout mice exhibited structurally physiological and **indiscriminate** from wild-type corneas (Nees et al., 2002). Nevertheless, a number of studies provide compelling evidences of the importance of ALDH3A1 for the maintenance of corneal clarity, especially under UVR exposure conditions. For example, Downes et al. (1994) reported that the ALDH3A1 null mouse strain SWR/J was susceptible to extensive corneal hazing after UVB exposure (Downes et al., 1994). Accordingly, a study by Lassen et al. (2007) evaluating the ocular phenotype of an *Aldh3a1* (-/-) knock-out mouse strain showed that ALDH3A1 deficient mice developed marked opacities in their cortex, along with enhanced cataract formation by one month of age. Additionally, after UVB exposure,

1-3 months old *Aldh3a1* (-/-) mice also exhibited increased anterior lens subcapsular opacities compared to wild-type mice. Interestingly, biochemical analysis revealed that the ocular opacities of the *Aldh3a1* (-/-) mice resulted from increased MDA- and 4-HNE- protein adduct levels and decreased proteasome activity (Lassen et al., 2007).

ALDH3A1 could arrest or retard corneal opacification by detoxifying MDA and 4-HNE, recycling glutathione, inhibiting the aggregation of oxidized and/or damaged proteins through a chaperone holdase activity or even by directly absorbing UV radiation. Experimental *in vitro* studies on the effect of UVB on recombinant human ALDH3A1, initially by Manzer et al. (2003) and later by Estey et al. (2010) revealed that UVB exposure causes structural transitions of ALDH3A1 through both covalent and non-covalent post-translational modifications that result in the formation of inactive, non-native, soluble ALDH3A1 aggregates with no evident precipitation (Estey et al., 2010; Manzer et al., 2003). The UV-absorption properties of ALDH3A1 could contribute to preventing the development of corneal precipitates through a mechanism of “suicide response” (Estey et al., 2010). In addition, this stress-dependent oligomerization and structural transition of ALDH3A1 is characteristic for proteins with holdase chaperone activity, like α -crystallin (Raman and Rao, 1997). Along these lines, Estey et al. (2007) demonstrated that ALDH3A1 partially unfolds and consequently loses its native tertiary structure also under thermal stress conditions. However, in this study ALDH3A1 failed to prevent the thermal aggregation of lactate dehydrogenase *in vitro* and managed to prevent the UV-induced inactivation of G6PD only at relatively high concentrations (Estey et al., 2007). In contrast, our experiments showed that both the MBP and 6xHis fusion ALDH3A1 recombinant proteins displayed significant efficiency in maintaining the enzymatic activity of *SmaI* after incubation at 37°C for 90 min. Furthermore, the 6xHis-tagged ALDH3A1 was able to

prevent the heat-induced aggregation and inactivation of CS. The discrepancies observed between the two studies could be attributed to the utilization of different target proteins, the usage of different relative concentrations of ALDH3A1 or even the diverse experimental conditions used.

According to our results, 6xHis-tagged ALDH3A1 showed to have enhanced chaperone-like activity in comparison to the MBP-tagged ALDH3A1. In addition, human 6xHis-tagged ALDH3A1 was also shown previously to exhibit higher specific enzymatic activity when compared to MBP-tagged ALDH3A1, most likely due to the large size of the MBP tag and its conformational impact on the proper folding of ALDH3A1 (Voulgaridou et al., 2013). However, the possibility that the enzymatic activity of ALDH3A1 contributes to the chaperone-like function of the protein has proved to be invalid as indicated by our experiments with the catalytically mutant form of 6xHis-tagged ALDH3A1. Dot heat shock assays revealed that expression of both wild-type and mutant 6xHis-tagged ALDH3A1 results in higher colony formation efficiencies in BL21(DE3) *E. coli* cells following incubation for 1 h at 65°C. The same observations were confirmed with thermotolerance assays, where expression of both wildtype and mutant human 6xHis-tagged ALDH3A1 was associated with increased tolerance to thermal stress.

Additionally, we were prompted to validate the protective effect of ALDH3A1 by utilizing a human corneal model. Cornea is located in the anterior segment of the eye and thus, is constantly exposed to various environmental stressors that affect the cellular oxidative balance and consequently lead to cellular oxidative stress (Cejka and Cejkova, 2015). Considering that oxidative stress significantly affects protein folding and induces the aggregation of proteins, through their oxidation, we investigated the effect of ALDH3A1 over-expression on the viability of the human corneal epithelial

HCE-2 cells under oxidative stress conditions (Nita and Grzybowski, 2016). Our results demonstrated that the expression of ALDH3A1 significantly inhibited the cytotoxic effects of both tert-butyl hydroperoxide and H₂O₂ on HCE-2 cells.

In summary, the present study showed that ALDH3A1 displays significant chaperone-like activity *in vitro*, as well as by utilizing bacterial and mammalian experimental models under the influence of thermal and/or oxidative stress. Nevertheless, future studies need to validate the chaperone-like potential in mammalian corneal epithelial cells further, under variable stress conditions. Furthermore, the study of ALDH3A1 surface hydrophobic anisotropies will allow us to determine whether the mechanisms by which the chaperone activity of ALDH3A1 is accomplished share any similarities with those of α -crystallin. Future research will undoubtedly reveal new insights into the currently unknown mechanisms of the chaperone function of ALDH3A1.

5. Acknowledgments

Funding: This research has been co-financed by the European Union (European Social Fund-ESF) and Greek national funds through the Operational Program “Education and Lifelong Learning” of the National Strategic Reference Framework (NSRF) – Research Funding Program: Heracleitus II. Investing in knowledge society through the European Social Fund.

FIGURE LEGENDS

Figure 1. Protective effect of ALDH3A1 upon thermal denaturation of *SmaI*.

A. Time-course of thermal inactivation of *SmaI* at 37°C and 42°C. One unit per reaction of *SmaI* in 1x of *SmaI* buffer was incubated at 37°C or 42°C for 30, 60 and 90 min. The residual activity of *SmaI* was assayed by the addition of 200 ng plasmid DNA in the

reaction mixture and its further incubation at 30°C for 90 min. **B & C.** Thermal denaturation of *SmaI* in the presence of ALDH3A1 at 37°C. Each reaction contained 1 unit *SmaI* and different concentrations of recombinant human ALDH3A1 in a total volume of 14 μ l of 1x *SmaI* buffer. The reaction was pre-incubated at 37°C for 90 min followed by incubation at 30°C for 90 min following the addition of 200 ng DNA. **B.** Recombinant MBP-tagged ALD3A1 (ALDH3A1/MBP) displayed protective effect against *SmaI* thermal inactivation at concentrations ≥ 53.3 nM. **C.** Recombinant 6xHis-tagged ALDH3A1 (ALDH3A1/6xHis) displayed protective effect against *SmaI* thermal inactivation at concentrations ≥ 53.3 nM. Control reactions are included showing plasmid DNA digestion performed at 30°C and 37°C respectively. Column buffer (CB; 1 μ l) and elution buffer (EB; 1 μ l) used in the purification of the ALDH3A1/MBP and ALDH3A1/6xHis protein respectively were included as additional controls. Digestions were run on 1% agarose gels and stained with ethidium bromide. M; Marker: λ DNAHindIII fragments, CB: column buffer, EB: elution buffer. Arrows indicate the size of λ DNAHindIII fragments.

Figure 2. Effect of MBP, BSA, lysozyme and denatured ALDH3A1 on thermal inactivation of *SmaI*.

A. Thermal denaturation of *SmaI* in the presence of MBP at 37°C. *SmaI* (1 unit) along with 1 μ l of various concentrations of recombinant MBP were diluted in a total volume of 14 μ l of 1x *SmaI* buffer. The mixture was then pre-incubated at 37°C for 90 min and subsequently incubated at 30°C for 90 min following the addition of 200 ng DNA. Recombinant MBP did not exhibit any protective effect against thermal denaturation of *SmaI* restriction enzyme. **B.** Thermal denaturation of *SmaI* in the presence of BSA, lysozyme and thermally inactivated 6xHis-tagged ALDH3A1. Each reaction contained 1 unit of *SmaI* and different concentrations of BSA, lysozyme and denatured

recombinant ALDH3A1 ranging from 0.053 to 1.6 μ M in a total volume of 13 μ l of 1x *SmaI* buffer. The recombinant 6xhis-tagged ALDH3A1 protein (ALDH31/6xHis) was previously denatured by boiling at 100°C for 10 min. For the thermal inactivation of *SmaI*, reactions were pre-incubated at 37°C for 90 min. *SmaI* residual activity was monitored by the addition of 200 ng of DNA and subsequent incubation at 30°C for 90 min.

Figure_3. Effect of ALDH3A1 on thermal aggregation of citrate synthase. Thermal aggregation of citrate synthase was carried out in the presence of recombinant ALDH3A1 at 43°C. Citrate synthase (75 μ g) was incubated at 43°C alone (*solid circles*) or in the presence of **A.** 62.5 nM (*open circles*), 125 nM, (*solid triangles*), 250 nM (*open triangles*) or 500 nM (*solid squares*) MBP-tagged ALDH3A1 (ALDH3A1/MBP) or **B.** 125 nM (*open circles*) and 500 nM (*closed triangles*) 6xHis-tagged ALDH3A1 (ALDH3A1/6xHis). ALDH3A1 appears to significantly prevent thermal aggregation of citrate synthase in a dose-dependent manner. At least three independent experiments were performed. Results from a representative experiment in which aggregation was monitored by measuring A_{360} are shown. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.01$.

Figure 4. Effect of ALDH3A1 on thermal inactivation of citrate synthase. Citrate synthase (100ng) was incubated at 37°C in the presence (*solid circles*) or absence (*open circles*) of 70 nM recombinant **A.** MBP-tagged ALDH3A1 (ALDH3A1/MBP) or **B.** 6xhis-tagged ALDHA3A1 (ALDH3A1/6xHis). CS activity was measured at various time points and expressed in the diagram as the % percentage of the control sample activity. At least three independent experiments were performed. Results from a representative experiment are shown. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.01$.

Figure 5. Protective effect of ALDH3A1 expression on the colony formation efficiency of *E. coli* cells under thermal stress conditions. **A.** *E. coli* cells transformed with the pet26b, pet26b/ALDH3A1 or pet26b/ALDH3A1^{mut} vectors were examined for their ability to survive and form colonies following an hour of incubation at 65°C and subsequent overnight recovery at 37°C. BL21(DE3) cells expressing either the wild-type or mutant 6xHis-tagged ALDH3A1 protein exhibited a significant survival advantage against thermal stress. At least three independent experiments were performed. **B.** Thermotolerance of *E. coli* cells expressing ALDH3A1. *E. coli* cells expressing either the wild-type (pET26b(+)/ALDH3A1 or mutant ALDH3A1 (pET26b(+)/ALDH3A1^{mut} cells) were examined for their ability to survive under different thermal stress conditions. Both wild-type and mutant ALD3A1 expression were associated with a survival advantage after incubation at **i.** 42°C for 3 hours, **ii.** 47.5°C for 1 hour, and **iii.** 52°C for 1 hour. Columns indicate the survival numbers of transformed *E. coli* cells without (black bars) and with ALDH3A1 induction, either in its wild-type (grey bars) or catalytically mutant (white bars) form. Data are shown as mean ± S.E (n=3). * $p < 0.05$, ** $p < 0.01$.

Figure_6. Establishment of the HCE-2 isogenic cell pair differing in the expression of human ALDH3A1. **A.** Western blot analysis of ALDH3A1 expression: Lane 1-2: 30 µg cell extracts, 1: ALDH3A1/HCE-2 cells, 2: mock/HCE-2 cells **B.** Comparison of ALDH3A1 mRNA levels between ALDH3A1 & mock HCE-2 cells by real time PCR **C.** Enzymatic activity of ALDH3A1 in ALDH3A1/HCE-2 and mock/HCE-2 cells. **B & C.** ALDH3A1/HCE-2 are represented by grey bars, while mock/HCE-2 cells are represented by black bars. Results from three independent experiments are shown. Results are expressed as mean ± S.E. *** $p < 0.001$.

Figure 7: Effect of tert-butyl hydroperoxide and hydrogen peroxide on cell viability of ALDH3A1/HCE-2 and mock/HCE-2 cells. ALDH3A1/HCE-2 (*solid circles*) and mock/HCE-2 cells (*open circles*) were exposed to increasing concentrations of **A.** tert-butyl hydroperoxide for 72 h and **B.** hydrogen peroxide for 72 h. Viability curves of ALDH3A1/HCE-2 cells are shifted to the right compared to mock/HCE-2 cells indicating increased tolerance of the ALDH3A1-expressing cells to the cytotoxic effects of the agents used. EC₅₀ values (half maximal effective concentrations) are indicated. Results are expressed as mean ± S.E. of three independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$

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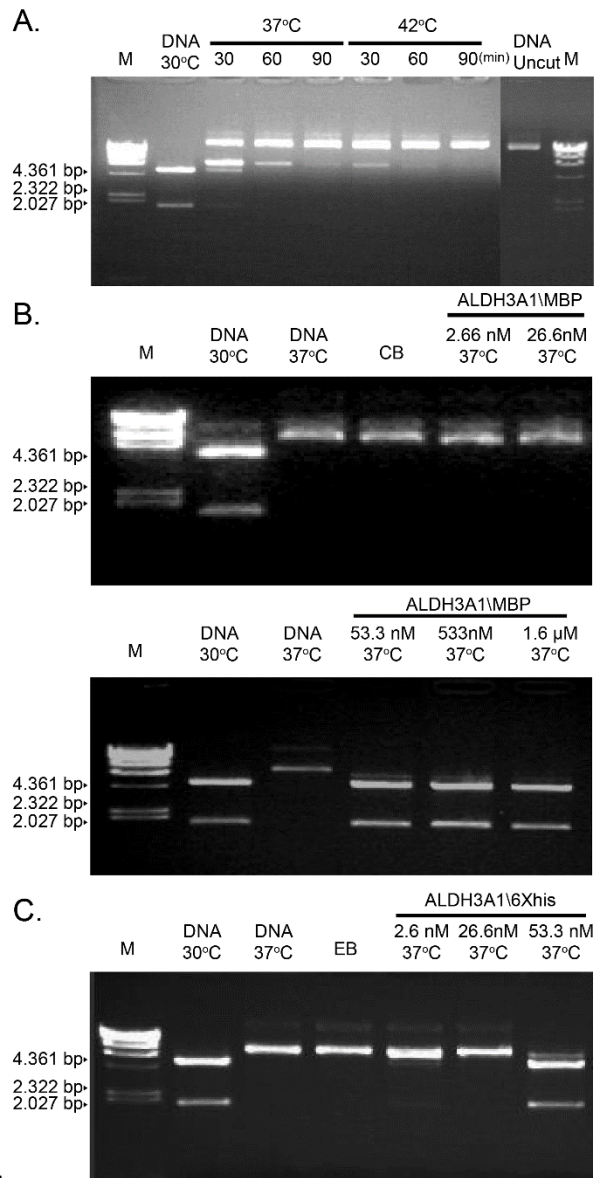
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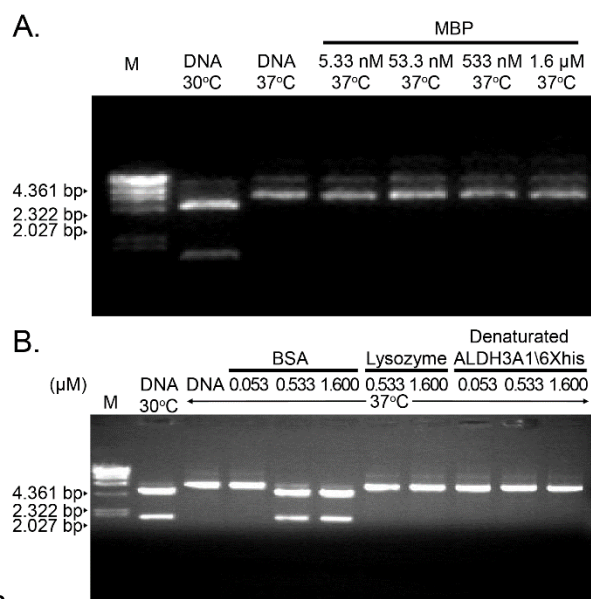
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Figure Caption



Figr-1



Figr-2

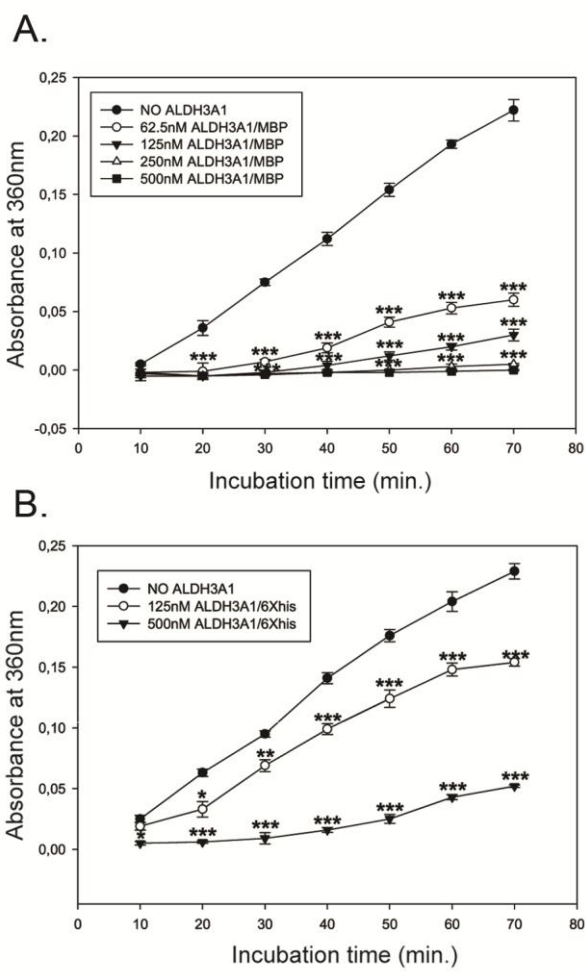
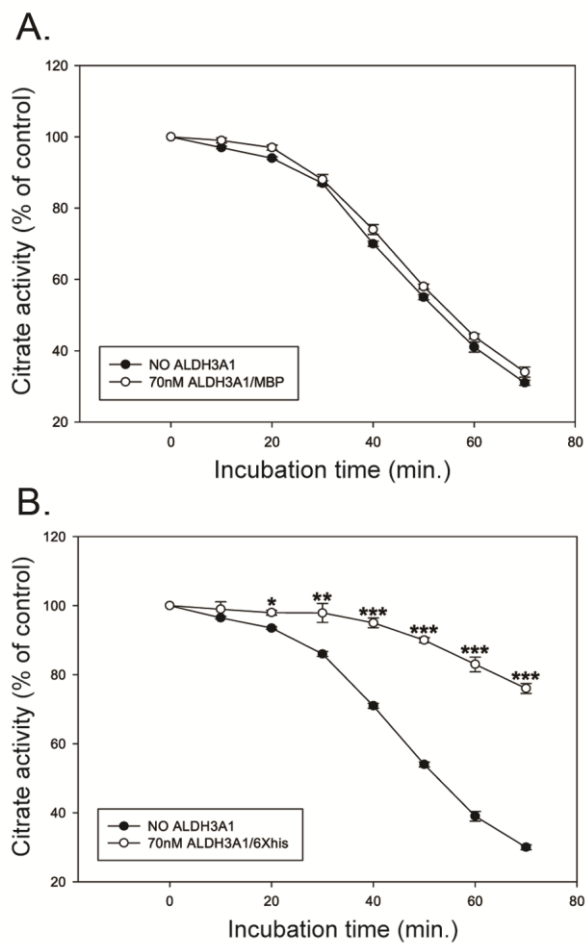


Fig-3



Figr-4

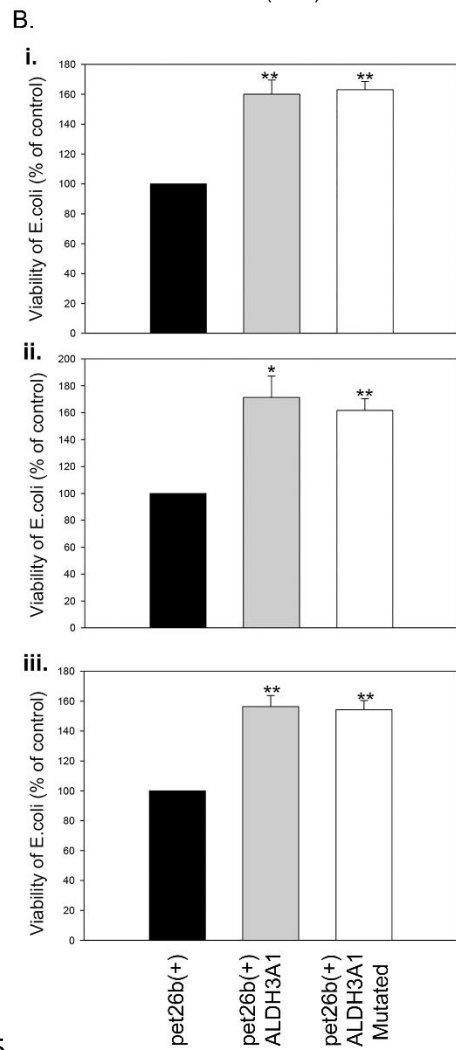
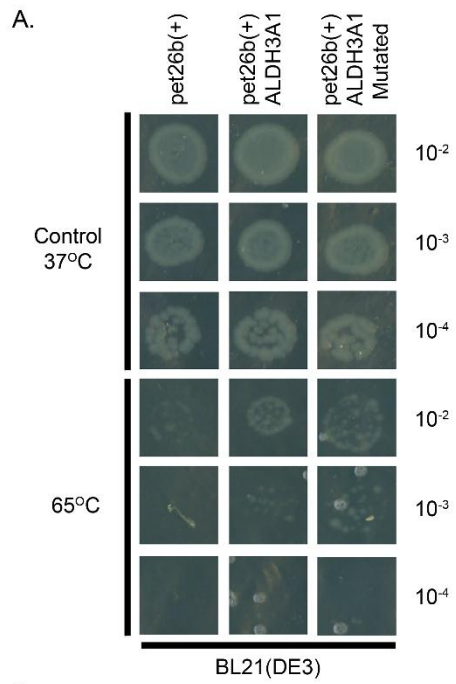


Fig-5

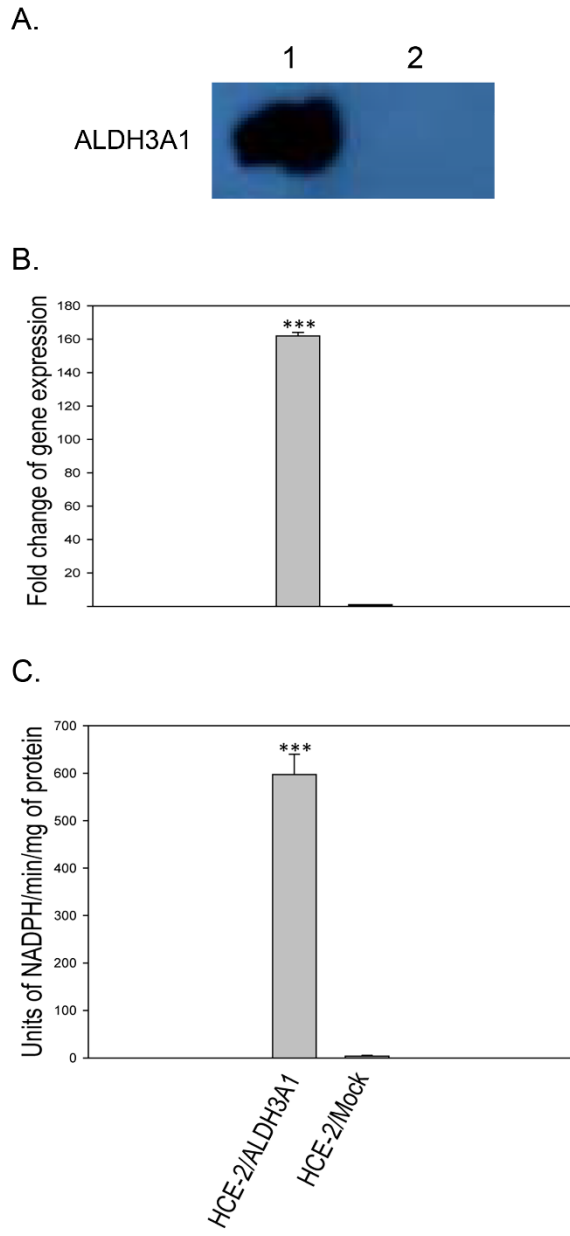


Fig-6

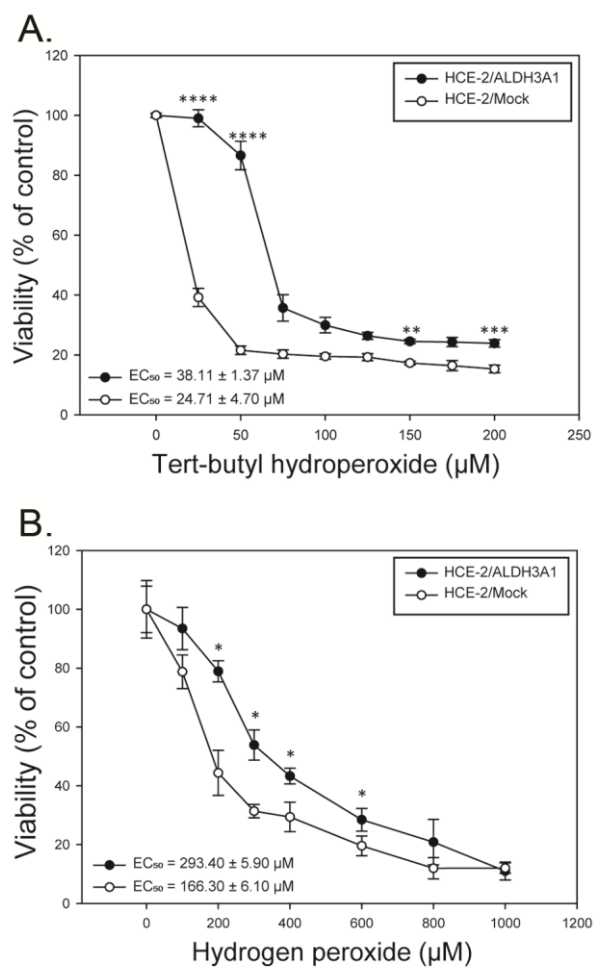


Fig-7