Title

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Endogenous ergothioneine is required for wild type levels of conidiogenesis and conidial survival but does not protect against 254 nm UV-induced mutagenesis or kill

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ABSTRACT

Ergothioneine, a histidine derivative, is concentrated in conidia of ascomycetous fungi. To investigate the function of ergothioneine, we crossed the wild type *Neurospora crassa* (Egt^+) and an ergothioneine non-producer (Egt^-, Δ*egt-1*, a knockout in NCU04343.5) and used the Egt^+ and Egt^- progeny strains for phenotypic analyses. Compared to the Egt^+ strains, Egt^- strains had a 59% reduction in the number of conidia produced on Vogel's agar. After storage of Egt^+ and Egt^- conidia at 97% and 52% relative humidity (RH) for a time course to either 17 or 98 days, respectively, Egt^- strains had a 23% and a 18% reduction in life expectancy at 97% and 52% RH, respectively, compared to the Egt^+ strains. Based on a Cu(II) reduction assay with the chelator bathocuproinedisulfonic acid disodium salt, ergothioneine accounts for 38% and 33% of water-soluble antioxidant capacity in *N. crassa* conidia from seven and 20 day-old cultures, respectively. In contrast, ergothioneine did not account for significant ($\alpha = 0.05$) anti-oxidant capacity in mycelia, which have lower concentrations of ergothioneine than conidia. The data are consistent with the hypothesis that ergothioneine has an antioxidant function *in vivo*. In contrast, experiments on the spontaneous mutation rate in Egt^+ and Egt^- strains and on the effects of 254 nm UV light on mutation rate and conidial viability do not support the hypothesis that ergothioneine protects DNA *in vivo*.

1. Introduction

The amino acid L-ergothioneine (2-mercaptohistidine trimethylbetaine, EGT) was first reported in 1909 in the ergot-producing fungus, *Claviceps purpurea* (Tanret, 1909). In the 1920s, EGT was recognized as a normal constituent in erythrocytes and blood in multiple animals including herbivores; EGT concentration in blood was demonstrated to be diet-dependent. In the 1950s, Melville (1959) showed that EGT is concentrated in certain tissues in animals, is synthesized by only some microbes including filamentous fungi, and is not synthesized by either animals or plants. In 2005, Gründemann et al. (2005) demonstrated that EGT is concentrated in cells that express an EGT-specific transporter, formerly named OCTN1 and now SLC22A4 in humans. SLC22A4 is highly expressed in erythrocyte progenitor cells, monocytes, fetal liver and bone marrow, brain neurons, the small intestine, trachea, kidney, cerebellum and lung (Gründemann, 2012; Kato et al., 2010). EGT is particularly concentrated in lens of the eye (Shires et al., 1997). In cells that express SLC22A4, such as during erythropoiesis, EGT can be at 1 mM (Hartman, 1990; Gründemann, 2012). The conservation of SLC22A4 homologs throughout the vertebrates and the concentration and retention of dietary EGT in particular tissues remains the strongest evidence that EGT has a function in animal physiology.

EGT biosynthesis is apparently limited to fungi except in the Saccharomycotina (Genghof, 1970; Bello et al., 2012), and to some actinomycetes (Genghof, 1970), mycobacteria (Genghof and Vandamme, 1964; Seebeck, 2010), some cyanobacteria (Pfeiffer et al., 2011), and the slime mold *Physarum polycephalum* (Genghof, 1970). *In vitro* assays with EGT support the hypothesis that it has intracellular antioxidant activity against selected reactive oxygen species (ROS) including hydroxyl radicals (·OH), hypochlorous acid (HOCl) and peroxynitrite (ONOO–) (Hartman, 1990; Akanmu et al., 1991; Aruoma et al., 1999; Hand and Honek, 2005; Franzoni et al., 2006; Cheah and Halliwell, 2012; Gruber et al., 2013). In contrast

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to glutathione, EGT is primarily present as a thione rather than a thiol at a physiological pH of 7.5 and consequently is resistant to auto-oxidation (Hartman, 1990; Hand and Honek, 2005). Partly because most of the work on EGT has been *in vitro*, the physiological role of EGT remains unestablished (Cheah and Halliwell, 2012).

In ascomycetous fungi, EGT is concentrated in conidia (Bello et al., 2012); *Neurospora crassa* conidia have ~5× more EGT per mg of soluble protein than mycelia and *Colletotrichum graminicola* conidia have ~17× more EGT than mycelia. Bello et al. (2012) identified the first EGT biosynthesis gene in a fungus, *egt-1* (*N. crassa* gene NCU04343) based on the EGT biosynthetic pathway in a mycobacterium (Seebeck, 2010); a Δ*egt-1* strain has no detectable EGT in either conidia or mycelia. Pluskal et al. (2014) demonstrated that the *Schizosaccharomyces pombe* homolog SPBC1604.01 was similarly required for EGT biosynthesis and that a second gene designated *egt2* (SPBC660.12c) also was involved in the biosynthetic pathway. Bello et al. (2012) showed that growth of the Δ*egt-1* mutant is indistinguishable from the wild type in the rate of hyphal elongation in Vogel's medium either with or without ammonium nitrate, and in the rate of germination of macroconidia on Vogel's medium. Similarly, the *S. pombe* EGT-deficient strain had no observed phenotypic defects during vegetative growth or quiescence (Pluskal et al., 2014). However, analysis of the lifespan of Δ*egt-1* conidia indicated that EGT has a function in maintenance of conidial viability during the quiescent period between conidiogenesis and germination. EGT also helped protect conidia during the germination process from the toxicity of exogenous peroxide radicals but apparently not from exogenous superoxide, singlet oxygen or Cu^{+2} (Bello et al., 2012).

The *egt-1* homologs in fungi have a unique set of three protein domains that allows unambiguous identification of homologs: an S-adenosyl methionine-dependent methyltransferase domain with a domain of unknown function (DUF) 2260; a DinB_2 domain with a HX_3HXE iron (II) binding motif; and a formylglycine-generating sulfatase domain (Seebeck, 2010; Bello et al., 2012). *egt-1* homologs are present in all of the true fungal groups in which EGT has been identified: the Pezizomycotina (i.e., Eurotiales, Glomerellales, Hypocreales, and Sordariales) and the Taphrinomycotina (i.e., Schizosaccharomycetales) in the Ascomycota; the Agaricomycotina (i.e., Agaricales, Boletales, and Cantharellales) and the Pucciniomycotina (i.e., Sporidiobolales) in the Basidiomycota; and the Mucoromycotina (i.e., Mucorales) (Melville, 1959; Genghof, 1970: Dubost et al., 2007; Ey et al., 2007; Bello et al., 2012). The *egt-1* homolog is absent in the Saccharomycotina, which do not produce EGT (Genghof, 1970; Bello et al., 2012). Although EGT has not been identified in any lower fungi except in the Mucoromycotina, *egt-1* homologs are present in a full genome-sequenced Blastocladiomycota and a Chytridiomycota (Bello et al., 2012). An *egt-1* homolog also is present in *Glomus intraradices* in the Glomeromycota (synonym: *Rhizophagus irregularis*, Joint Genome Institute, USA).

In addition to a postulated antioxidant role, EGT has been postulated to have cytoprotective effects including against UV and γ-radiation (Cheah and Halliwell, 2012) and some mutagens (Hartman and Hartman, 1987). Interestingly, the EGT biosynthetic gene *egt-1* was annotated as a late light responsive gene (Chen et al., 2009; Wang et al., 2012). Here, we produced isogenic Egt^+ and Egt^- strains, and demonstrated that the absence of EGT in conidia (1) reduces the number of conidia by approximately one-half, (2) reduces the life-span of conidia by approximately one-fifth at both 97% and 52% relative humidity, (3) does not affect the mutation rate in the absence of an exogenous mutagen and (4) does not appear to affect the rate of 254 nm UV-induced kill or mutagenesis. A total antioxidant capacity assay on extracts indicates that EGT accounts for one-third of the antioxidant capacity in conidia.

2. Materials and methods

2.1. Fungal culture

Strains were maintained in silica gel at −70 °C (Bello et al., 2012). Except when indicated otherwise, conidia were produced in slants with Vogel's medium (1× Vogel's salts, 15 g sucrose, 2 g ammonium nitrate and 15 g agar/l), and harvested either dry or as indicated in Bello et al. (2012). Mycelia were produced in stationary Vogel's broth in the dark for 24 h and harvested as described previously (Bello et al., 2012).

2.2. Generation of Egt^+ and Egt^- strains

N. crassa wild type ORS-6a (FGSC 4200, *mat a*) (Perkins, 2004) and the knockout mutant of the EGT biosynthetic gene Δ*egt-1::hph* (locus NCU04343, FGSC 19115, *mat A*, derived from FGSC 2489) were obtained from the Fungal Genetics Stock Center (Colot et al., 2006). A genetic cross between the Δ*egt-1* as female (Egt^- $hygromycin^R$) and wild type strain (Egt^+ $hygromycin^S$) was performed following a Fungal Genetics Stock Center protocol (www.fgsc.net). Forty colonies derived from ejected ascospores were randomly selected and screened for hygromycin resistance. Putative hygromycin resistant colonies were re-screened.

DNA was isolated from the two parental and 40 ascospore-derived strains and used for PCR analysis with *egt-1*-specific primers (forward 5′-CACGCTTACAACGGTTTGTG-′3, reverse 5′-AGCTGGAAA CCTCAAGCAAG-′3), which amplify 500 bp from bp 951 to bp 1450 from the start codon of gene NCU04343.5, and *hph*-specific primers (forward 5′-GTCCTGCGGGTAAATAGCTG-′3, reverse 5′-ACATTGTTG GAGCCGAAATC-′3), which amplify 472 bp of the hygromycin phosphotransferase gene. All 22 hygromycin-resistant colonies, including the parental Δ*egt-1* were PCR-positive for the hygromycin resistance gene and PCR-negative for the *egt-1* gene, and all the hygromycin-sensitive colonies, including the parental wild type were PCR-negative for the hygromycin resistance gene and PCR-positive for the *egt-1* gene. Six of the Δ*egt-1* and six of the *egt-1*$^+$ ascospore-derived strains were randomly selected for determination of EGT (Section 2.7) and other experiments.

2.3. Quantification of conidial production

Fifty-ml tissue culture flasks (25 cm^2 and a loosely closed, vented screw cap; Corning Inc., Corning, NY) containing 10 ml Vogel's agar were inoculated with a mycelial plug near the mouth of the flask, and incubated at 30 °C in the dark. After 24 h, when the mycelia covered the agar, flasks were incubated at 30 °C, 15 cm below continuous fluorescent lights (Phillips Coolwhite F20T12-CW 20 W). After 7 days, conidia were collected in two successive incubations in 15 ml 0.1% Tween 20 on a rotary shaker at 140 rpm for 5 min. Hyphal fragments were removed by pouring the suspension through two layers of Miracloth. Conidial production was estimated with a hemocytometer. The experiment was conducted in a completely randomized design in three independent trials, with one replicate per strain per trial. To achieve homoscedasticity for ANOVA, conida/cm^2 were log-transformed.

2.4. Storage at high (97%) and low (52%) relative humidity (RH)

Conidia were retrieved from seven-day-old slants, placed in open 1.5 ml microfuge tubes, sealed in a scintillation vial containing 6.5 ml of a saturated solution of either potassium sulfate or magnesium nitrate for 97% or 52% RH, respectively (Wexler and Hasegawa, 1954) and stored in the dark at 30 °C for a time course

to either 17 or 98 days. To assess germination, conidia were suspended in water, adjusted in concentration, and deposited in a 50 μl drop onto a dish with water agar. After incubation for 6 h at 28 °C, conidia were killed with lactophenol cotton blue (20% lactic acid, 20% phenol, 40% glycerol, and 0.0025% cotton blue in water) and then assessed microscopically. Percentage germination was based on 100 conidia in each of two determinations per replicate. Ascospore progeny data are based on six Egt^+ and six Egt^- strains as replicates in each of two trials for each RH. Parental strains were only examined at 97% RH as three replicates. Logistic curves were fitted to percentage germination as a function of days in storage using DAP (www.gnu.org/software/dap, GNU, Free Software Foundation). The average life expectancy was computed as the area under the curve and the median time to death (TD_{50}) was calculated as the predicted day in which 50% of the conidia were dead.

2.5. Exposure of conidia to 254 nm UV: survival and mutation rate

To assess percentage kill, conidia from the parental strains and the six Egt^+ and Egt^- progeny strains were harvested from slants. Conidia (2.5×10^5) suspended in a 50 μl 0.1% Tween 20 droplet were placed on water agar dishes (60 × 15 mm). Immediately, lidless dishes were irradiated with 254 nm UV light at either 0, 100, 200, 300, or 400 J/m^2 with a UV crosslinker (Fisher Scientific FB-UVXL-1000). After treatment, the dishes were incubated at 30 °C in the dark for 6 h. Conidia were stained with lactophenol cotton blue and percentage germination was assessed for 100 conidia per replicate. The experiment was conducted and evaluated in a completely randomized design in three independent trials for the wild type and Δ*egt-1* knockout, and in two trials with the six Egt^+ and Egt^- ascospore progeny as replicates. Fraction germinated data were arcsine square-root transformed to achieve homoscedasticity for ANOVA.

To determine mutation rates at the *mtr* (methyltryptophan resistance) locus, which encodes an amino acid permease, we followed Dillon and Stadler's (1994) protocol. Briefly, Petri dishes (100 × 15 mm) contained 15 ml Vogel's agar amended with 20 g sorbose and 30 mg *p*-fluorophenylalanine (FPA; Sigma–Aldrich, St. Louis, MO) per liter. Conidia were dispensed onto dishes and then assessed for two response variables: *mtr* mutation rate and germination rate. To assess the *mtr* mutation rate at 0 and 50 J/m^2, for each replicate, 1×10^7 conidia were spread onto each of ten and three dishes, respectively. For 100 J/m^2 replicate, 5×10^6 conidia were spread onto each of 3 dishes. After incubation of dishes at 30 °C for 72 h, FPA-resistant colonies were counted. To assess the germination rate, 50 μl droplets of conidial suspensions were placed onto water agar plates and irradiated as above. After incubation for 6 h at 30 °C in the dark, conidia were stained and percentage germination was assessed for 100 conidia per replicate. The experiment was conducted in a completely randomized design in five independent trials for the wild type, Δ*egt-1* knockout, and two Egt^+ and Egt^- ascospore progeny. Data were analyzed by ANOVA with trial as block. Specific comparisons were done by contrast analysis.

2.6. Total antioxidant capacity assay

Conidia were collected from slants, suspended in 0.1% Tween 20, counted with a hemocytometer and distributed as 3×10^8 conidia per 2 ml cryovial tube. Two-hundred and fifty μl 0.5-mm-diameter glass beads (BioSpec, Bartlesville, OK) were added to each tube of either conidia or mycelia. After freezing and lyophilization, cells were broken in a Fast-Prep (BioSpec) with seven, 30 s rounds of shaking interspersed with 1 min on ice. After addition of 600 μl phosphate buffered saline (pH 7.5), 50 μl of dichloromethane (DCM, Sigma–Aldrich) was added per tube to remove the lipophilic contents; after gentle mixing, the DCM extract was concentrated in the bottom of the tubes by centrifugation at 16,000*g* for 7 min. The aqueous phase was used to determine the antioxidant capacity with the chelator bathocuproinedisulfonic acid (OxiSelect™ Total Antioxidant Capacity Kit, Cell Biolabs, San Diego, CA); uric acid was used as an antioxidant standard (Da Cruz, 2003; Campos et al., 2009). Total antioxidant capacity was normalized to soluble protein using the bicinchoninic acid (BCA) assay with an albumin standard (Pierce/Thermo Scientific, Rochester NY). Germinability of the non-extracted conidia was monitored to assure cell viability; after 6 h, germination in all strains was >90%, with no significant ($\alpha = 0.05$) effects of either an EGT group * culture age interaction, EGT group or culture age. The experiment was conducted in a completely randomized design in three independent trials with six Egt^+ and six Egt^- ascospore progeny. Data were analyzed by ANOVA with trial as block.

2.7. Quantification of conidial EGT and reduced glutathione (GSH) by reverse-phase HPLC-fluorescence detection

Conidia were harvested from tissue culture flasks as indicated above. Lyophilized conidia were disrupted with a metal pestle in a 1.5 ml tube containing 250 μl of 0.5-mm-diameter glass beads while the tubes were on dry ice. Then 550 μl ice-cold 10 mM Tris pH 8.5 was added and the tube was gently mixed. A 50 μl aliquot with cell debris was transferred with a wide-bore pipette tip (2 mm diameter), diluted 1:10 in 10 mM Tris pH 8.5, and incubated for 30 min at 37 °C. After conducting the BCA reaction for protein, the supernatant was clarified with DCM before quantifying absorbance.

A 120 μl-aliquot of the cell extract was also removed for EGT and GSH quantification. After clarification of cell extracts with DCM as indicated above, either the cell-free supernatant or a standard dilution series were derivatized using monobromobimane (mBBr; THYOLITE, Calbiochem, La Jolla, CA) (Fahey and Newton, 1987) essentially as described in Bello et al. (2012) with the following modifications. Ten μl of mBBr (1 mg/ml in acetonitrile) was added to 90 μl of cell-free lysate in a dark tube, and the mixture was briefly vortexed. After incubation at 24 °C for 3 h, the pH of the mixture was adjusted to 3.0–3.5 with 2.5 μl of 85% phosphoric acid to stop the reaction. The experiment was conducted in a completely randomized design with three independent biological replicates, with each replicate conducted as a separate trial.

3. Results

3.1. Generation of Egt^+ and Egt^- strains

To investigate the function of EGT, isogenic lines of wild type (Egt^+) and the knockout in gene NCU04343.5 (Δ*egt-1::hph*) (Colot et al., 2006) were crossed. The forty assayed progeny had the expected 1:1 segregation of *egt-1*$^+$ to *hph* ($\chi^2 = 0.1$, $P = 0.7$) with 100% co-segregation of hygromycin-sensitivity with *egt-1*$^+$ and hygromycin-resistance with *hph*. Six *egt-1*$^+$ and six Δ*egt-1* progeny were randomly selected (Fig. 1) and screened for EGT and GSH (data not shown). EGT was not detected in either the parental knockout or the six progeny Δ*egt-1* strains. EGT was detected in all the *egt-1*$^+$ strains. There were no significant differences of either EGT ($P = 0.52$) or GSH ($P = 0.39$) concentrations in conidia of the wild type parent or the six Egt^+ progeny strains.

3.2. Egt^- strains produce significantly fewer conidia than Egt^+ strains

The number of conidia produced by the two parental strains, and the six Egt^+ and six Egt^- ascospore progeny were determined

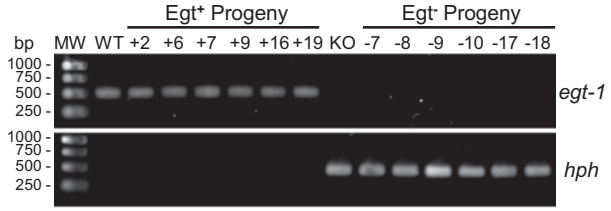

Fig. 1. Agarose gel of PCR amplicons of the ergothioneine (EGT) biosynthetic gene *egt-1* and the hygromycin resistance gene *hph* from *N. crassa* strains: the wild type parent (WT, Egt^+ $hygromycin^S$); the insertionally mutagenized knockout parent (KO, Egt^- $hygromycin^R$); and six of their ascospore progeny that either produced (Egt^+) or did not produce ergothioneine (Egt^-) by HPLC. MW, molecular mass markers with the indicated number of bp.

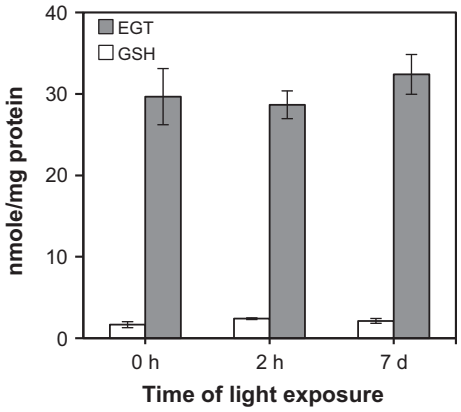

Fig. 2. The effect of either constant darkness or light exposure for either 2 h or 7 days on the concentration of ergothioneine (EGT) and reduced glutathione (GSH) in wild type *N. crassa* conidia. Error bars are ± SE.

(Table 1). In an ANOVA with the six progeny as replicates, the Egt^- strains had significantly fewer conidia than the Egt^+ strains in three independent trials ($P = 0.011$, 0.008, and 0.0006). Similarly, the parental Egt^- produced significantly ($P = 0.022$) fewer conidia than the wild type. In an analysis of EGT group with trial as block, there was no significant block effect ($P = 0.22$) and a highly significant EGT effect ($P = 0.0002$). In a Dunnett's multiple comparison procedure ($\alpha = 0.05$) with the wild type as the control, each of the Egt^- strains had significantly fewer conidia ($P = 0.0007$–0.04) and none of the Egt^+ strains differed significantly ($P = 0.52$–1.0) from the wild type. Based on the three trials, the detransformed mean of the Egt^+ ascospore progeny was 3.6×10^5 conidia/cm^2 with a detransformed 95% confidence interval (CI_{95}) from 2.9×10^5 to 4.6×10^5 conidia/cm^2, and the Egt^- progeny had a mean of 1.5×10^5 conidia/cm^2 with a detransformed CI_{95} from 1.2×10^5 to 1.8×10^5 conidia/cm^2. Consequently, the Egt^- strains had a 59 ± 6 % (±SE) reduction in the number of conidia, and produced only 41 ± 6% of the conidia in the Egt^+ strains.

3.3. Exposure to light during conidiation does not affect EGT concentration in conidia

After wild type colonized Vogel's agar in the dark for 24 h, the dishes were either incubated in the dark for 7 days, for 2 h under the lights and then the remainder of 7 days in the dark, or under continuous light for 7 days. An ANOVA of the concentration of EGT and GSH in conidia (Fig. 2) indicated that there were no significant differences between light treatments on either EGT ($P = 0.61$) or GSH ($P = 0.25$) concentrations in conidia. Consequently, EGT synthesis occurs in the dark and is apparently light-independent.

Table 1
Effect of genotype on the number of conidia produced by strains of *N. crassa* that produce (Egt^+) or do not produce (Egt^-) ergothioneine (EGT)[a].

Strain type	Egt^+ strains		Egt^- strains	
	Strain name	Log (conidia/cm^2) ± SE[b]	Strain name	Log (conidia/cm^2) ± SE[b]
Parental	Wild type	5.7 ± 0.04	FGSC 19115	5.3 ± 0.03
Progeny	+2	5.6 ± 0.05	−7	5.2 ± 0.8
Progeny	+6	5.5 ± 0.09	−8	5.2 ± 0.07
Progeny	+7	5.5 ± 0.16	−9	5.1 ± 0.17
Progeny	+9	5.8 ± 0.13	−10	5.2 ± 0.13
Progeny	+16	5.5 ± 0.12	−17	5.0 ± 0.10
Progeny	+19	5.5 ± 0.11	−18	5.2 ± 0.08

[a] Progeny are from ascospores of a Δ*egt-1* (Egt^-) × wild type (Egt^+) cross.
[b] In an ANOVA with progeny as replicates, the Egt^- progeny had significantly fewer conidia than the Egt^+ progeny in three trials with $P = 0.011$, 0.008, and 0.0006. The knockout also produced significantly ($P = 0.022$) fewer conidia than the wild type.

3.4. Egt^- conidia have significantly reduced longevity compared to Egt^+ conidia at both high (97%) and low (52%) relative humidity (RH)

Conidia from seven-day-old slants were stored in the dark at 30 °C at either 97% (Fig. 3A) or 52% (Fig 3B) RH for a time course to either 17 or 98 days, respectively. Assessments of percentage germination were used to fit logistic curves and to estimate the average life expectancy and the median time to death (TD_{50}) (Table 2). Egt^- strains had a highly significantly ($P < 0.001$) reduced life expectancy with a 23 ± 8 and an 18 ± 3% reduction during storage at 97% and 52% RH, respectively. Life expectancies and TD_{50} were highly correlated ($r = 0.9999$, $P < 0.0001$).

3.5. Endogenous EGT does not affect the mutation rate in untreated conidia and does not mitigate the lethality or the mutagenicity of 254 nm UV light

Egt^+ and Egt^- conidia were either untreated or treated with 254 nm UV light at 100–400 J/m^2, and assessed for the percentage germination (Fig. 4). In a regression analysis of conidial germination as a function of UV dose, there was no significant interaction of UV dose * EGT group ($P = 0.55$), a highly significant effect of UV dose ($P < 0.0001$), but no significant effect of EGT group ($P = 0.59$). Consequently, the presence or absence of endogenous EGT in the conidia does not affect the lethality of 254 nm UV light, and EGT does not have a protective effect against 254 nm UV-induced kill.

The mutation rate at the *mtr* locus was assessed as the rate to FPA resistance (Fig. 5). In a randomized complete block design with trial as block, there was no significant trial effect ($P = 0.22$), a significant UV intensity * strain interaction ($P = 0.037$), a highly significant UV intensity effect ($P < 0.0001$), and a highly significant EGT group effect ($P < 0.0001$). However, while the untreated parental Δ*egt-1* conidia had a significantly higher mutation rate than the untreated wild type ($P = 0.001$), the two Egt^- progeny strains did not have an increased mutation rate compared to wild type ($P = 0.07$), and were significantly ($P < 0.001$) less than the parental Δ*egt-1*. Similarly the parental Δ*egt-1* treated with 50 Joules/m^2 has a higher mutation rate ($P = 0.006$) than the wild type but the two Egt^- progeny strains did not have a significantly higher mutation rate ($\alpha = 0.05$) than the wild type. Consequently, the absence of EGT does not result in an increased mutation rate to FPA resistance, either without or with a pretreatment of 254 nm UV light.

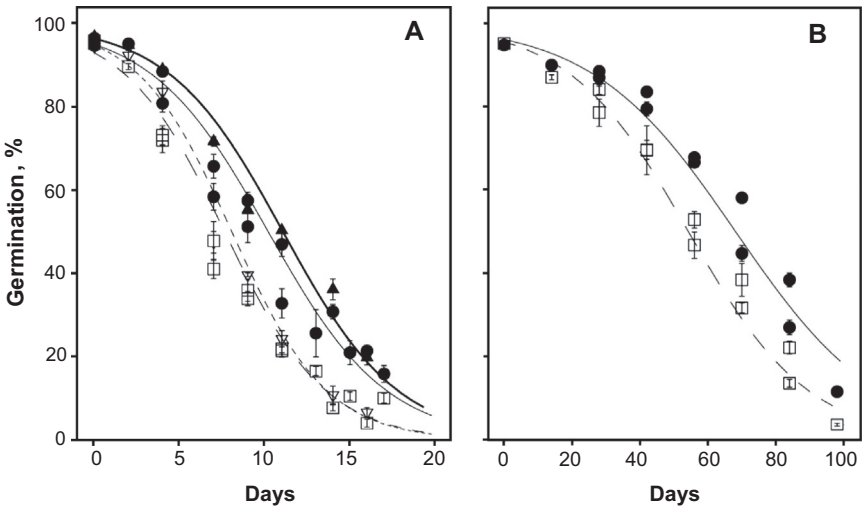

Fig. 3. Effect of endogenous ergothioneine (EGT) on the percentage germination over time of *N. crassa* conidia stored at either (A) 97% relative humidity (RH) or (B) 52% RH. The points are the means ± SE of six Egt$^+$ (●) and six Egt$^-$ (□) progeny strains from two trials. Fitted logistic curves are shown for the Egt$^+$ (—), and Egt$^-$ (– –) strains. In (A), data (n = 3) are also shown for the two parental strains: Egt$^+$ (▲), and Egt$^-$ (△).

Table 2
Effect of endogenous ergothioneine (EGT) on survival of *N. crassa* conidia in storage in the dark at 30 °C at either 97% or 52% relative humidity (RH)[a].

Strain type	RH in storage, %	Phenotype			
		Egt$^+$	Egt$^-$	Egt$^+$	Egt$^-$
		Life expectancy ± SE, days[b,c,d]		TD_{50} ± SE, days[b,c,d]	
Parental	97	12 ± 0.1	8.7 ± 0.1	11 ± 0.05	8.4 ± 0.4
Progeny	97	11 ± 0.2	8.4 ± 0.2	11 ± 0.2	8.0 ± 0.2
Progeny	52	71 ± 0.8	58 ± 0.7	69 ± 0.7	56 ± 0.8

[a] Conidia were collected from seven-day-old cultures and then stored at the indicated RH.
[b] Life expectancy and time to death of the median conidium (TD_{50}) were estimated from the fitted logistic curves shown in Fig. 3. Estimates of progeny are based on two trials, each with six Egt$^+$ and six Egt$^-$ strains, and estimates of parental strains are based on three replicates.
[c] Within each row, for each response variable, ANOVA contrasts indicated that Egt$^-$ strains survived for significantly ($P < 0.001$) shorter periods than those the Egt$^+$ strains.
[d] For each response variable at 97% RH, ANOVA contrasts indicated that the wild type and Egt$^+$ progeny were not significantly different and that the parental Δ*egt-1* and the Egt$^-$ progeny were not significantly different ($\alpha = 0.05$).

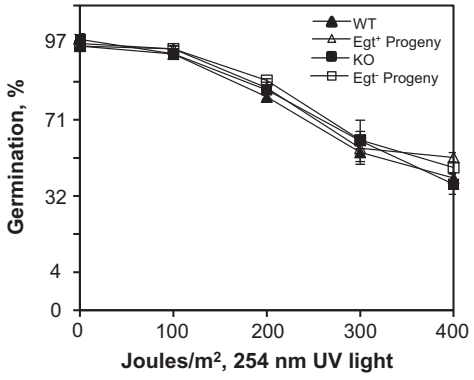

Fig. 4. Effect of endogenous ergothioneine (EGT) on the lethality of 254 nm UV light, as assessed by the percentage germination of *N. crassa* conidia after exposure of 0–400 J/m^2. Means ± SE of an arcsine square root transformation of the fraction germinated are shown, with detransformed values on the y axis. Values are from three trials of the parental strains (WT, Egt$^+$) and (KO, Egt$^-$) (n = 3), and two trials of six Egt$^+$ and six Egt$^-$ ascospore progeny (n = 12).

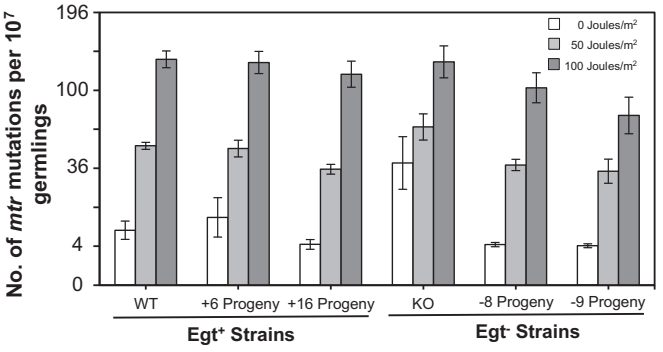

Fig. 5. Effect of endogenous ergothioneine (EGT) on the mutation rate to *p*-fluorophenylalanine (FPA) resistance at the *mtr* locus after exposure to 0, 50 and 100 J/m^2 of 254 nm UV light. Means ± SE of a square-root transformation of the number of FPA-resistant colonies per 10^7 conidial germlings are shown, with detransformed values on the y axis. Data are from five trials (n = 5) with the two parental strains (WT and KO) and two Egt$^+$ and two Egt$^-$ progeny.

3.6. Endogenous EGT accounts for approximately one-third of water-soluble antioxidant capacity in conidia, but no significant antioxidant capacity in mycelia

A copper reduction-based assay with the chelator bathocuproinedisulfonic acid was used to estimate antioxidant capacity in conidial and mycelial extracts (Table 3). Based on hemocytometer-adjusted counts of conidia from seven and 20-day-old cultures, there was no age * EGT group interaction (P = 0.6), and highly significant ($P < 0.0001$) EGT group and age effects on antioxidant capacity. Based on the three trials, EGT accounts for 38 ± 6 (±SE) and 33 ± 6% of the water-soluble antioxidant capacity in *N. crassa*

Table 3
Effect of endogenous ergothioneine (EGT) on total antioxidant capacity (TAC) of seven and 20-day-old *N. crassa* conidia and one-day-old mycelia[v].

EGT group[w]	Cells	nmoles uric acid equivalents of TAC per 10^7 conidia[x]	nmoles uric acid equivalents of TAC per mg soluble protein[x,y]
+	7-day-old conidia	186 a	601 a
–	7-day-old conidia	117 b	386 b
+	20-day old conidia	141 b	380 bc
–	20-day old conidia	94 c	262 c
+	Mycelia	NA[z]	113 d
–	Mycelia	NA	146 d

[v] Total antioxidant capacity of aqueous extracts were determined with a Cu(II) reduction assay with the chelator bathocuproinedisulfonic acid disodium salt and a uric acid standard.
[w] Each of three trials had six progeny isolates per EGT group in a completely randomized design.
[x] Data from were log-transformed to achieve homoscedasticity for ANOVA, and then analyzed with trial as block. Within a column, values followed by the same letter are not significantly different by Tukey's HSD, $\alpha = 0.05$. Detransformed means are shown.
[y] Soluble protein was determined by the bicinchoninic acid method.
[z] NA, not applicable.

conidia from seven and 20 day-old cultures, respectively. Analysis of estimates of antioxidant capacity based on solubilized protein from conidia from seven and 20-day-old cultures produced similar statistical results with no age $*$ EGT group interaction ($P = 0.12$), and highly significant ($P < 0.0001$) EGT group and age effects. Conversely, there were no significant differences ($\alpha = 0.05$, Tukey's HSD) between the antioxidant capacity in the Egt^+ and Egt^- strains in the one-day-old mycelia.

4. Discussion

The *N. crassa* isogenic Egt^+ and Egt^- strains provide an *in vivo* model for investigating the function(s) of EGT in eukaryotic cells. Here, we demonstrated that conidia without EGT have a ≈20% shorter life expectancy than those with EGT, regardless of whether they are stored at 30 °C in a relatively low relative humidity (52%), which allows a relatively long life in storage with a median of 71 days, or at a high relative humidity (97%) with a median lifespan of only 11 days in storage, after a seven-day period for conidial production. Munkres and Furtek (1984) identified a 22-day median lifespan (including conidial production) for wild type *N. crassa* conidia incubated at 30 °C under white light in 85–100% RH. Previously, Bello et al. (2012) estimated a 30% reduction in conidial life span in the original Δ*egt-1* strain, but only the two parental strains were examined and the relative humidity was not controlled. In the fission yeast *S. pombe* (Taphrinomycotina), glucose-starved cells had increased concentrations of trehalose and EGT, an extended lifespan, and increased resistance to 40 mM H_2O_2 (Pluskal et al., 2011). ROS-induced damage has been closely associated with cell aging in numerous studies (Sigler et al., 1999; Gruber et al., 2013). Munkres et al. (1984) demonstrated that *N. crassa* mutants with defective antioxidant enzymes (superoxide dismutase, catalase, glutathione peroxidase, cytochrome c peroxidase and ascorbate free radical reductase) had decreased conidial lifespans; these mutants would be expected to have had increased concentrations of endogenous ROS in their conidia. In the plant pathogenic filamentous fungus *Alternaria brassicicola* and the animal pathogen *Aspergillus fumigatus*, genetic disruption of *TmpL*, which encodes for a protein that affects redox homeostasis, results in an increased oxidative burst during conidiation, accelerated aging, and greater sensitivity to oxidative stress (Kim et al., 2009). Conversely, a decreased production of ROS in mitochondria was associated with an increased lifespan in the filamentous ascomycete *Podospora anserina* (Osiewacz, 2002).

Although all aerobic organisms are exposed to endogenous ROS from metabolism, conidia have to withstand a variety of abiotic stresses that cause increased ROS, including dehydration, heat, UV light, ozone, salts and heavy metals (Munkres, 1992; Mittler and Zilinskas, 2004). Based on a copper-reduction assay as an estimate of total antioxidant activity normalized per mg soluble protein, EGT accounts for 38 ± 0.4% of the water-soluble antioxidants in young conidia from seven-day-old cultures but a non-significant percentage of the antioxidant capacity in young (one day old), non-conidiating mycelia. Bello et al. (2012) estimated that wild type *N. crassa* conidia and mycelia (per mg soluble protein) contain 25.4 ± 1.3 (±SE) nmol EGT and 5.2 ± 0.4 nmol EGT, respectively, and 3.0 ± 0.1 nmol GSH and 5.5 ± 0.1 nmol GSH, respectively. In contrast, the Δ*egt-1* strain had no detectable EGT but similar quantities of GSH as the wild type. The significant ($\alpha = 0.05$) contribution of EGT to the antioxidant capacity in the conidia but not in the mycelia is consistent with the 5× concentration in conidia. Expected water-soluble antioxidants other than EGT and GSH include mannitol, proline, trehalose, ascorbate, and uric acid (Aguirre et al., 2005; Chen and Dickman 2005; Ruijter et al., 2003). Overall, the one-third reduction in total antioxidant capacity in conidial extracts of Egt^- compared to Egt^+ strains and the 20% reduction of conidial of lifespan suggest that EGT has an antioxidant role *in vivo* and that EGT has a direct role in conidial survival.

The Egt^- strains produced only half of the conidia produced by Egt^+ strains. A strain of the ascomycete *Oidiodendron maius* with a knockout of a superoxide dismutase gene produced more ROS, was more sensitive to compounds that cause oxidative stress, and produced only 60% of the conidia compared to the wild type (Abbà et al., 2009). *N. crassa* mutants in superoxide dismutase were also hypersensitive to oxidants and were female-sterile (Munkres, 1992). Hansberg and colleagues have proposed that the transition from an undifferentiated state, such as a mycelium, to a differentiated state, such as a conidium, and conversely from the differentiated state of a conidium to an undifferentiated germling involves an oxidative burst (Toledo et al., 1995; Aguirre et al., 2005). Whether EGT is involved in maintaining the requisite concentration of ROS in order for conidiogenesis to occur remains to be determined. Interestingly, Ishimoto et al. (2014) suggest that in mouse neural progenitor cells, OCTN1-mediated uptake of EGT both suppresses cell proliferation by regulation of oxidative stress and promotes cellular differentiation by modulating expression of transcription factors.

Here, conidia produced in complete darkness had the same concentration of EGT as those produced after exposure to either 2 h or 7 days of light. Although light stimulates *N. crassa* conidiation (Greenwald et al., 2010), cultures kept in the dark do produce conidia, albeit at one-quarter the level of those incubated in light (Lauter et al., 1997). Transcription of *egt-1* is increased in the presence of light (Chen et al., 2009; Wang et al., 2012) and there is decreased expression of *egt-1* in the white-collar mutants WC-1 and WC-2, which encode for transcription factors associated with conidiogenesis (Schmoll et al., 2012). However, our data suggest that *egt-1* expression might be associated with conidiogenesis rather than light-induction *per se*.

Previously, there has been relatively little data on the function of EGT *in vivo*. Pluskal et al. (2014) estimated that the intracellular concentration of EGT in vegetative cells of *S. pombe* is 0.3 μM in the control, 42 μM in glucose-starved cells and 157 μM in nitrogen-starved cells. Microarray data with *S. pombe* indicate that the *egt-1* homolog is up-regulated 10-fold when cells are exposed to H_2O_2 (Chen et al., 2003). Paul and Snyder (2010) reduced OCTN1 by 75% in HeLa cells with a RNAi strategy; as a consequence,

transfected cells with reduced endogenous EGT had decreased cell proliferation and had significantly more mortality from H_2O_2 than control cells. Kato et al. (2010) produced mice with an *octn1*$^{-/-}$ knockout; the knockout mice had no quantifiable EGT in their tissues, in contrast to the wild type mice. Excised intestines from *octn1*$^{-/-}$ mice that were exposed to an ischemia–reperfusion treatment that induces ROS and inflammation had significantly more tissue mortality than tissue from wild type mice. As indicated above, some Actinobacteria, which do not produce GSH, produce EGT and mycothiol, which contains a cysteine residue with an acetylated amino group linked to a glucosamine which is then linked to inositol (Fahey, 2013). Surprisingly, a *Mycobacterium smegmatis* strain that is devoid of mycothiol because of a defect in the mycothiol biosynthesis gene *MshA*, has 35× more EGT during exponential growth than the wild type strain, indicating that EGT and mycothiol are co-regulated in a prokaryote (Ta et al., 2011).

Cheah and Halliwell (2012) reviewed the literature that suggest that EGT might protect against mutagenesis of DNA:EGT absorbs in the UV with a λ_{max} = 257 nm and ε_{max} = 14,000 M^{-1} cm^{-1} (Carlsson et al., 1974); EGT and OCTN1 are present in skin cells (Markova et al., 2009); UV damage involves ROS generation; and *in vitro*, EGT provides protection against UV-induced cell damage and death (Markova et al., 2009). Using the OCTN1-reduced vs. OCTN1-normal HeLa cells, Paul and Snyder (2010) demonstrated that, after a hydrogen peroxide treatment, there was more mitochondrial DNA damage in OCTN1-reduced than in the OCTN1-normal cells. However, here we demonstrated *in vivo* that endogenous EGT does not affect either the 254 nm UV light-induced *mtr* mutation rate or the lethality of 254 nm UV light in *N. crassa* conidia. In addition, in the absence of a UV-treatment, the endogenous EGT does not affect the mutation rate in the *mtr* locus in *N. crassa* conidia.

The original Δ*egt-1* knockout (FGSC 19115) had an increased mutation rate that was absent in the two Egt$^-$ progeny strains (Fig. 5). Consequently, we postulate that the original knockout has an additional mutation that is absent in at least the Δ*egt-1* progeny strains −8 and −9. Because neither of the two Egt$^-$ progeny strains tested have the increased UV-independent mutation rate, the additional mutation in FGSC 19115 is apparently unlinked to Δ*egt-1*. Since the high throughput knockout process in *N. crassa* starts with a strain defective in DNA repair with either Δ*mus-51::bar* or Δ*mus-52::bar* (Colot et al., 2006), we demonstrated that the knockout was sensitive to glufosinate ammonium, the *bar* selectable marker (data not shown). Thus, FGSC 19115 presumably had the wild type *mus* restored as intended in the Colot et al. (2006) protocol. In addition, Ninomiya et al. (2004) demonstrated that neither the *N. crassa* Δ*mus-51* nor the Δ*mus-52* strains were more sensitive to UV than the wild type, which also suggests that the unidentified mutation in FGSC 19115 is not in either *mus-51 or mus-52*.

Overall, the Egt$^-$ and Egt$^+$ progeny allow a functional analysis of EGT. To conclude, here we provide additional documentation of the effect of EGT on conidial longevity, demonstrate that EGT is involved in conidiogenesis and does not affect the endogenous mutation rate in conidia, and provide evidence that EGT functions as an antioxidant and not as a preventative of 254 nm-induced UV mutation or death.

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