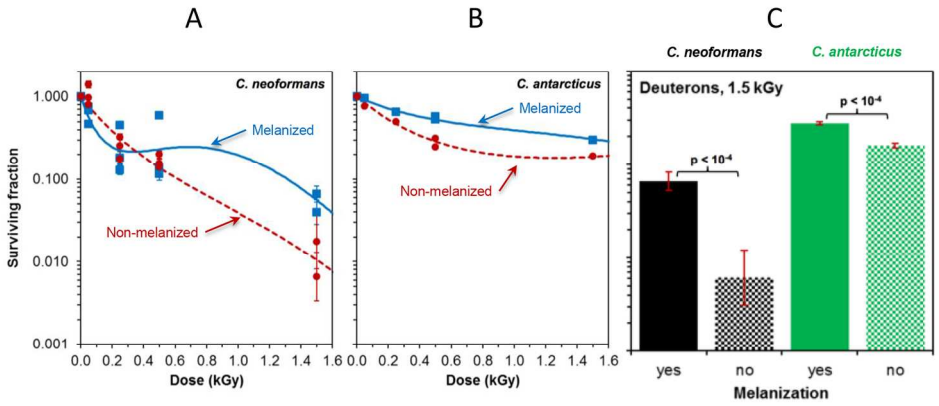

Fig. 1. Clonogenic survival of melanized and non-melanized CN and CA after deuteron irradiation: A) CN; B) CA. Symbols represent data and curves represent IR model fits. Several symbols at the same radiation dose and fungal strain represent data from different replicate experiments. Error bars represent 95% CIs. Blue = melanized, red = non-melanized; C) Comparison of clonogenic survival of melanized vs non-melanized strains of CN (black) and CA (green) after the highest tested doses of deuterons and X-ray irradiation. Error bars represent 95% CIs.

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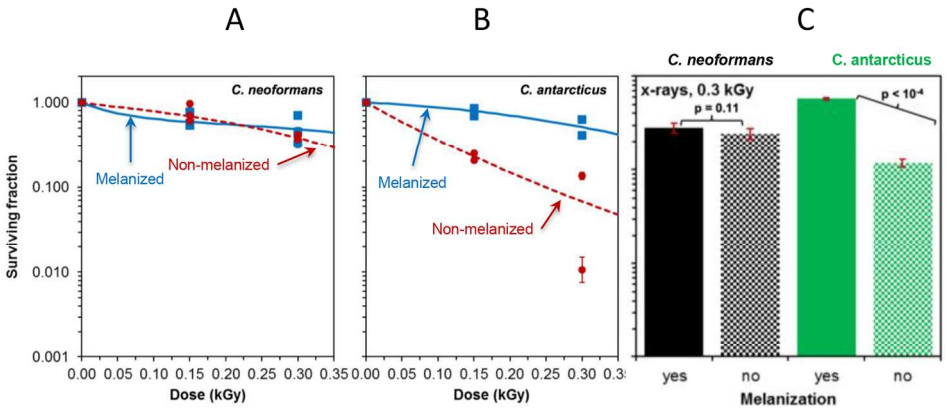

Fig. 2. Clonogenic survival of melanized and non-melanized CN and CA after X-ray irradiation: A) CN; B) CA. Symbols represent data and curves represent IR model fits. Error bars represent 95% CIs. Blue = melanized, red = non-melanized; C) Comparison of clonogenic survival of melanized vs non-melanized strains of CN (black) and CA (green) after the highest tested doses of deuterons and X-ray irradiation. Error bars represent 95% CIs.

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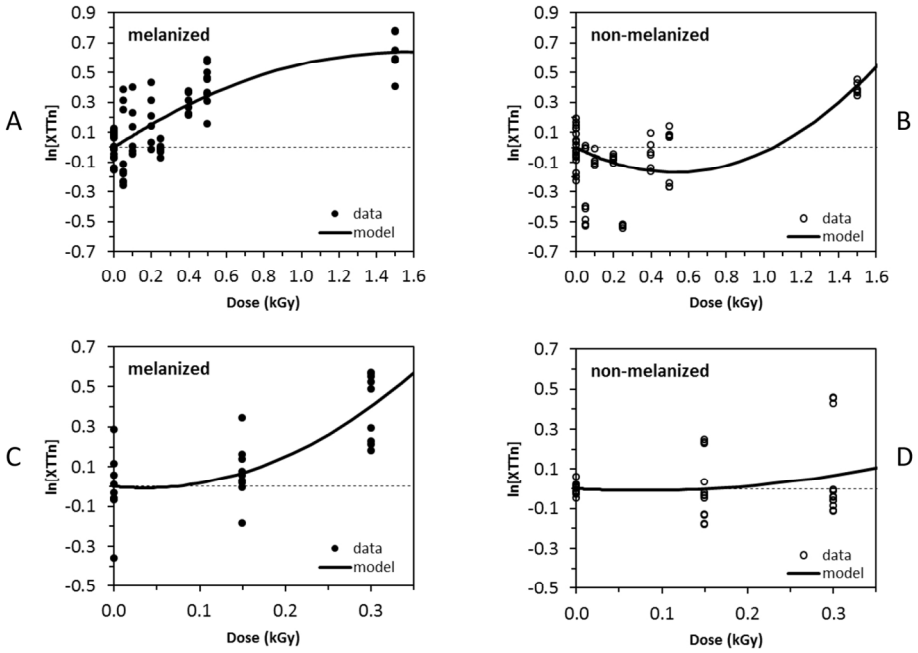

Fig. 3. Effects of irradiation on CN XTT reducing activity. A) Melanized cells exposed to deuterons. B) non-melanized cells exposed to deuterons. C) melanized cells exposed to X-rays. D) non-melanized cells exposed to X-rays. Closed symbols represent melanized cells, and open symbols represent non-melanized cells. The results were normalized by the number of cells per well and these values were again normalized by dividing by the control values which were the non-irradiated cells at time zero, n stands for normalized.

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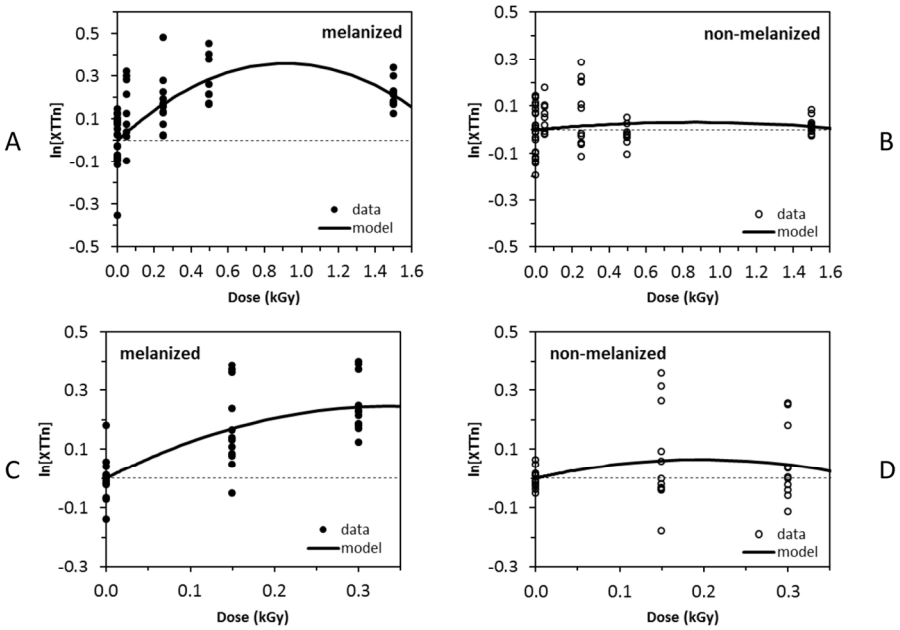

Fig.4. Effects of irradiation on CA XTT reducing activity. A) Melanized cells exposed to deuterons. B) non-melanized cells exposed to deuterons. C) melanized cells exposed to X-rays. D) non-melanized cells exposed to X-rays. Closed symbols represent melanized cells, and open symbols represent non-melanized cells. The results were normalized by the number of cells per well and these values were again normalized by dividing by the control values which were the non-irradiated cells at time zero, n stands for normalized.

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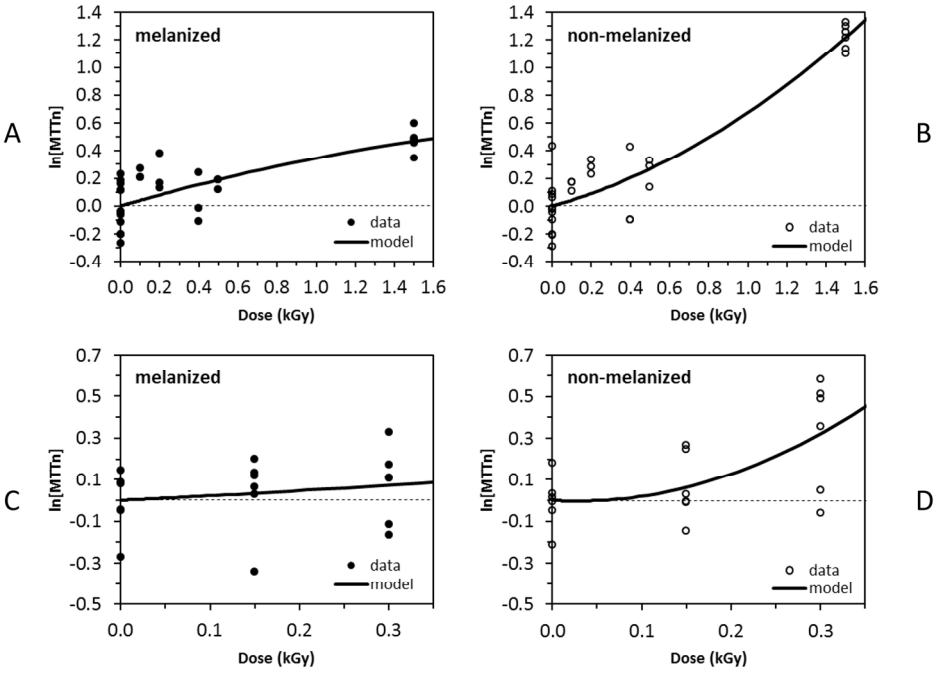

Fig. 5. Effects of irradiation on CN MTT reducing activity. A) Melanized cells exposed to deuterons. B) non-melanized cells exposed to deuterons. C) melanized cells exposed to X-rays. D) non-melanized cells exposed to X-rays. Closed symbols represent melanized cells, and open symbols represent non-melanized cells. The results were normalized by the number of cells per well and these values were again normalized by dividing by the control values which were the non-irradiated cells at time zero, n stands for normalized.

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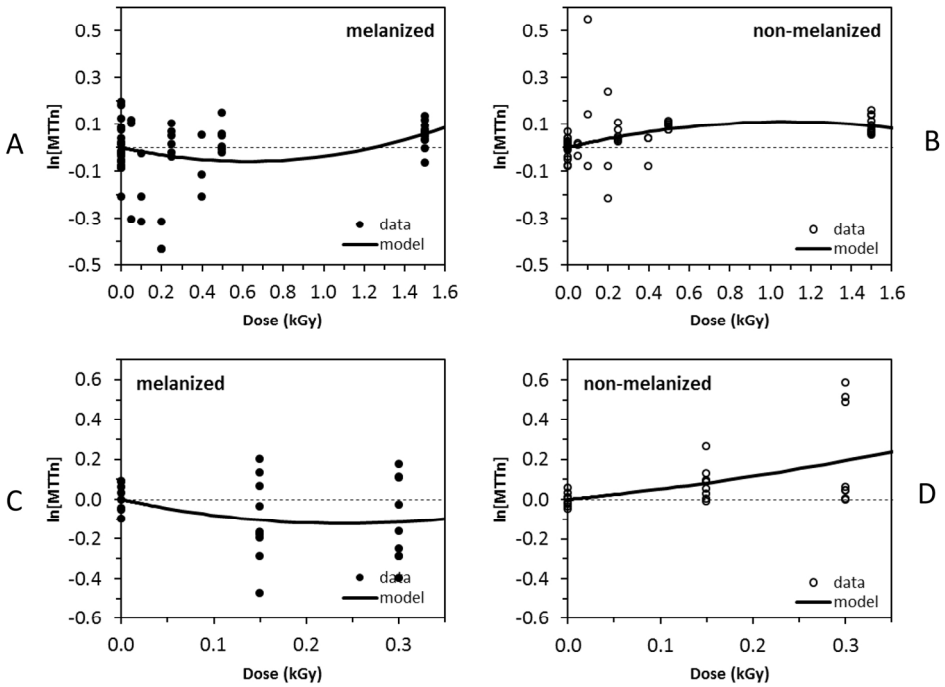

Fig. 6. Effects of irradiation on CA MTT reducing activity. A) Melanized cells exposed to deuterons. B) non-melanized cells exposed to deuterons. C) melanized cells exposed to X-rays. D) non-melanized cells exposed to X-rays. Closed symbols represent melanized cells, and open symbols represent non-melanized cells. The results were normalized by the number of cells per well and these values were again normalized by dividing by the control values which were the non-irradiated cells at time zero, n stands for normalized.

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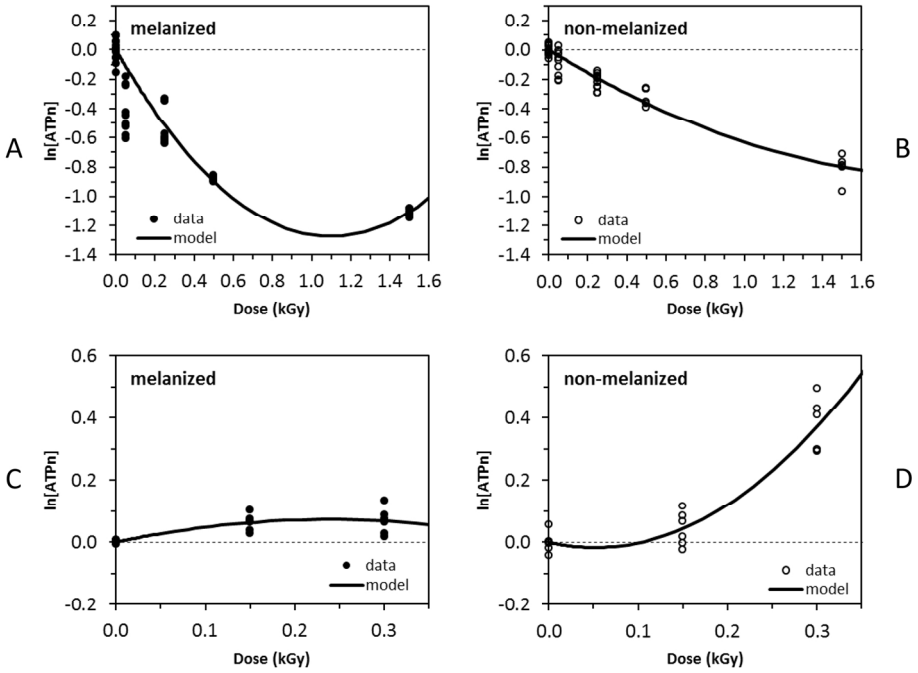

Fig. 7. Effects of irradiation on CN ATP level. A) Melanized cells exposed to deuterons. B) non-melanized cells exposed to deuterons. C) melanized cells exposed to X-rays. D) non-melanized cells exposed to X-rays. Closed symbols represent melanized cells, and open symbols represent non-melanized cells. The results were normalized by the number of cells per well and these values were again normalized by dividing by the control values which were the non-irradiated cells at time zero, n stands for normalized.

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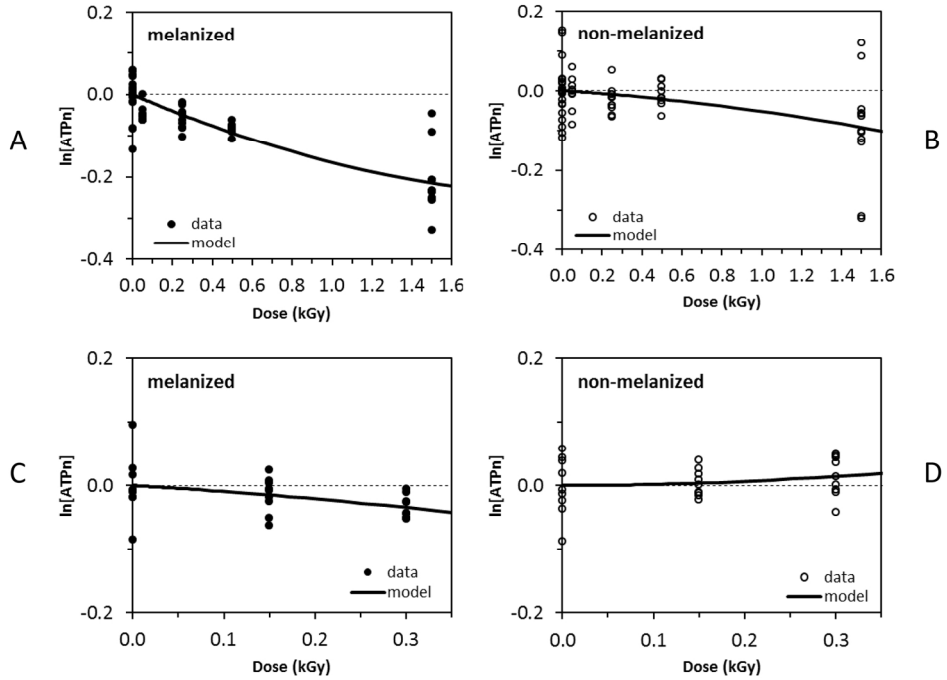

Fig. 8. Effects of irradiation on CA ATP level. A) Melanized cells exposed to deuterons. B) non-melanized cells exposed to deuterons. C) melanized cells exposed to X-rays. D) non-melanized cells exposed to X-rays. Closed symbols represent melanized cells, and open symbols represent non-melanized cells. The results were normalized by the number of cells per well and these values were again normalized by dividing by the control values which were the non-irradiated cells at time zero, n stands for normalized.

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Melanin is effective in protecting fast and slow growing fungi from various types of ionizing radiation

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Non-standard abbreviations: XTT (2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide); MTT (2-(4,5-dimethyl-2-thiazolyl)-3,5-diphenyl-2H-tetrazolium bromide); ATP (Adenosine triphosphate); Gy (Gray) ISS (international space station)

SUMMARY

Melanin is a ubiquitous pigment with unique physicochemical properties. The resistance of melanized fungi to cosmic and terrestrial ionizing radiation suggests that

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melanin also plays a pivotal role in radioprotection. In this study, we compared the effects of densely-ionizing deuterons and sparsely-ionizing X-rays on two microscopic fungi capable of melanogenesis. We utilized the fast-growing pathogenic basiodiomycete forming an induced DOPA-melanin, *Cryptococcus neoformans* (CN); and the slow-growing environmental rock-inhabiting ascomycete synthesizing – a constitutive DHN-melanin, *Cryomyces antarcticus* (CA); melanized and non-melanized counterparts were compared. CA was more resistant to deuterons than CN, and similar resistance was observed for X-rays. Melanin afforded protection against high-dose (1.5 kGy) deuterons for both CN and CA (p-values $<10^{-4}$). For X-rays (0.3 kGy), melanin protected CA (p-values $<10^{-4}$) and probably CN. Deuterons increased XTT activity in melanized strains of both species, while the activity in non-melanized cells remained stable or decreased. For ATP levels the reverse occurred: it decreased in melanized strains, but not in non-melanized ones, after deuteron exposure. For both XTT and ATP, which reflect the metabolic activity of the cells, ~~the~~ larger and more statistically-significant differences as a function of melanization status occurred in CN. Our data show, for the first time, that melanin protected both fast-growing and slow-growing fungi from high doses of deuterons under physiological conditions. These observations may give clues for creating melanin-based radioprotectors.

Keywords: black fungi, melanin, ionizing radiation, deuterons, X-rays

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INTRODUCTION

Melanin is a ubiquitous pigment in nature and possesses a variety of unique physical, chemical, paramagnetic and semiconductive properties (Meredith and Sarna, 2006). The melanin amount in *Cryptococcus neoformans (CN)* and other melanized fungi constitute 10-15% of the fungal cellular biomass dry weight (Wang *et al.,* 1996; Revskaya *et al.,* 2012). Many melanized fungi are very radioresistant, requiring radiation doses exceeding 5 kGy to reduce cell survival to 10% (Dadachova and Casadevall, 2008). Such doses are roughly 1000-fold higher than the lethal dose for humans, showing that extreme radioresistance is not limited to prokaryotes such as *Deinococcus radiodurans*, and can be achieved by eukaryotic cells. Moreover, since fungi are eukaryotes, the main features of their cellular machinery share important similarities with the human counterparts; this makes them better microbial models for human radiation responses than prokaryotes. The resistance of melanized fungi to cosmic radiation in the Mir Spacecraft and the presence of numerous melanized fungal species in the damaged nuclear reactor at Chernobyl suggest that melanins also play a pivotal role in protection from ionizing radiation (Vember *et al*., 1999; Novikova, 2004; Dadachova and Casadevall, 2008).

During the last decade, some important insights into the interaction of fungal melanin with ionizing radiation have been reported. One of the studies indicated that the radioprotective properties of fungal melanin result from a combination of physical shielding, chemical composition, spherical arrangement and free radical quenching (Dadachova *et al*., 2008) and another demonstrated that irradiation of melanin alters its oxidation-reduction potential (Turick *et al*., 2011). Radiotropism, the propensity of fungi to grow towards a radiation source, was described in the late 1990-s (Vember *et al.*

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1999). Since then,– increased growth of melanized fungi was demonstrated after irradiation with gamma-rays and X-rays (Dadachova *et al*., 2007; Robertson *et al*., 2012; Shuryak *et al*., 2014) and gamma-rays have been shown to alter the electronic properties of melanins (Dadachova *et al*., 2007; Khajo *et al*. 2011; Robertson *et al*., 2012; Shuryak *et al*., 2014). In addition, recent transcriptomic investigation revealed that ionizing radiation stimulates protein biosynthesis with input from melanin (Robertson *et al*., 2012). The radioprotective properties of melanins are of high interest for a number of different potential applications, including the development of novel and effective radioprotective materials for saving cancer patients undergoing radiation therapy from the side effects of ionizing radiation treatment, and protection of astronauts from space radiation in manned space missions. Data confirm that radioprotective properties of fungal melanins can be exploited in mammalian systems; in fact, it was recently reported that internal administration of melanin protected mice against experimental lethal irradiation (Schweitzer *et al*., 2010; Kunwar *et al*., 2012; Revskaya *et al*., 2012; Rageh *et al*., 2014).

Most research on radioresistance mechanisms and radioprotection involves the use of high-energy photons (gamma~~γ~~ and X-rays) which are sparsely ionizing, so that energy deposition and ionizations are relatively randomly distributed throughout the irradiated material (Sachs *et al*., 1997). However, more recently, densely ionizing ~~high energy~~ protons and heavy ions which produce a definitive dense track of radiation events in their path (Hada and Georgakilas, 2008; Stewart *et al*., 2011) are attracting interest both in the field of radiation oncology and for radiation protection in manned space missions (Durante, 2014). In this study, we compared the effects of ~~highly~~ densely ionizing

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deuterons and sparsely ionizing X-rays on two microscopic fungi capable of melanogenesis – the pathogenic basiodiomycete *CN*, which formsing an induced DOPA-melanin, *Cryptococcus neoformans* and the slow-growing and space-resistant environmental rock-inhabiting ascomycete which synthesizes a constitutive DHN-melanin *Cryomyces antarcticus* (*CA*), which synthesizes a constitutive DHN-melanin (Onofri *et al*., 2012; Onofri *et al*., 2015). In this study we investigated the effects of melanin on both fungal species exposed to both types of radiation.

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RESULTS

Protective effects of melanin on both fungal species.

The clonogenic survival of melanized and non-melanized *C. neoformansCN* and *C. antarcticusCA* after deuteron irradiation is shown in Fig. 1A and 1B, respectively. *Cryptococcus neoformansCN* H99 and its non melanized laccase deficient mutant Lac(-) were used to generate melanized and non-melanized cells, respectively, while To obtain non-melanized cells of *C. antarcticusCA*, tricyclazole [5-methyl-1,2,4-triazolo(3,4-b) benzothiazole] was added to cultures for inhibition of the DHN-melanin pathway, as melanin deficient mutants for this fungus are not available. The dData, which encompassed a wide range of doses (0-1.5 kGy), suggested that both melanized and non-melanized *C. neoformansCN* strains showed fairly high (>10%) survival at doses <0.5 kGy (Fig. 1A), but at 1.5 kGy, melanized cells survived much better than non-melanized ones. At this high dose of deuterons, melanization clearly resulted in increased survival of *C. neoformansCN* in all replicate experiments: the magnitude of this protective effect was quite large (10-fold) (Fig. 1C), and there was robust statistical significance (Table 2).

C. antarcticus

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CA was clearly more resistant to killing by deuteron radiation than ~~*C. neoformans*~~*CN* throughout the tested dose range (Fig. 1B). The melanized strain showed substantially higher survival at 1.5 kGy (Fig. 1C) than the non-melanized one, and this effect had robust statistical significance (Table 2).

Because of technical reasons, the dose ra~~nge~~te ~~delivered by~~for X-ray~~s~~ exposure ~~(0-0.3 kGy)~~ was much ~~narrower and~~ lower than the deuteron dose rate (Table 1), and therefore the maximum X-ray dose which could be delivered during an acceptable irradiation time was much lower than for deuterons (0.3 vs 1.5 kGy, respectively). However, the results revealed that in contrast to deuteron irradiation, non-melanized ~~*C. neoformans*~~*CN* was more resistant to X-rays than non-melanized ~~*C. antarcticus*~~*CA* (Fig. 2A, B). In fact, it was as resistant as melanized ~~*C. neoformans*~~*CN*. (Fig. 2C, Tables 2-3). For ~~*C. antarcticus*~~*CA*- the high sensitivity of the non-melanized strain to X-rays resulted in a statistically significant difference in survival between the melanized and non-melanized cells (Fig. 2C, Tables 2-3). Overall, these results show for the first time that in both ~~*C. neoformans*~~*CN* and ~~*C. antarcticus*~~*CA* the presence of melanin provided protection against high doses of deuterons.

Comparison of the IR and LQ models for cell survival~~.~~

The best-fit induced repair (IR) model prediction curves for deuteron-irradiated ~~*C. neoformans*~~*CN* and ~~*C. antarcticus*~~*CA* were consistent with the experimental data (Fig. 1). The survival curves for melanized vs. non-melanized strains of ~~*C. neoformans*~~*CN* diverged at the highest tested dose of 1.5 kGy. The melanized strain clearly survived this dose better~~,~~ than the non-melanized one (Fig 1A, C). A similar pattern was observed for

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~~*C. antarcticus*~~*CA* (Fig. 1–B, C). These melanization-dependent differences in survival were mathematically described by the best-fit parameter values for the IR model (Table 3). Overall, the IR model described the data ~~adequately,~~ much better than the simpler linear quadratic (LQ) model (by ≥14 sample size corrected Akaike information criterion units). However, the IR model is not the only plausible explanation for the shape of the dose responses we observed here.

Metabolic activity assays

XTT (2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide) and MTT (2-(4,5-dimethyl-2-thiazolyl)-3,5-diphenyl-2H-tetrazolium bromide) results demonstrate differences in metabolic activity for melanized vs non-melanized cells post irradiation. The use of XTT and MTT assays in parallel can help to define the location of the melanin-mediated electron transfer in the cells; positively charged MTT is taken into the cells via the plasma membrane potential and is reduced intracellularly while the negatively charged XTT is cell impermeable and its reduction occurs at the cell surface (Berridge *et al.*, 2005).

Our results (Fig. 3, Table 1S) suggest that deuteron irradiation of ~~*C. neoformans*~~*CN* increased XTT levels in melanized cells, but not in non-melanized ones. The effects of X-rays were less clear, probably due to limitations of the dose range, as previously mentioned. For *CN* ~~*neoformans*~~, XTT was increased in melanized cells but not in non-melanized cells at high radiation doses, ~~but the differences were not specific.~~ For ~~*C. antarcticus*~~*CA*, deuterons and X-rays increased XTT in melanized cells, but in non-melanized ones there was no statistically-significant change (Fig. 4, Table 1S). MTT

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levels in melanized cells were somewhat increased as a result of deuteron irradiation in non-melanized ~~*C. neoformans*~~*CN* cells and in melanized ones as well. However, the differences between irradiated and control were not statistically significant (both the linear and quadratic coefficients ~~are positive, but their~~had 95% CIs which overlapped zero; Fig. 5, Table 1S). ~~-~~MTT in ~~*C. antarcticus*~~*CA* also did not provide a clear pattern (Fig. 6, Table 1S) and~~but~~ overall there was not much change in MTT levels for either melanized or non-melanized cells.

Radiation exposure resulted in decreased ATP (adenosine triphosphate) level in melanized cells post irradiation~~.~~ (Figs. 7 and 8 and Table 1S). ~~display the changes in ATP concentration in melanized and non-melanized cells post irradiation.~~ Deuterons caused significant decreases in ATP levels in melanized ~~*C. neoformans*~~*CN* and ~~*C. antarcticus*~~*CA*, whereas the effect was weaker/inconclusive in non-melanized cells of both species (Figs. 7 and 8 and Table 1S). This is consistent with the report of decrease in ATP in melanized ~~*C. neoformans*~~*CN* cells post exposure to gamma~~-~~rays, UV and visible light (Bryan *et al*., 2011).

DISCUSSION

In this study we compared the effects of deuterons and X-rays on two microscopic fungi capable of melanogenesis – fast growing ~~*C. neoformans*~~*CN* (doubling time 2 hours) and slow growing ~~*C. antarcticus*~~*CA* (doubling time approximately 2 weeks). ~~*C. neoformans*~~*CN* is a ubiquitous soil dwelling microorganism able to survive at 37^{o}C, which makes it an opportunistic human pathogen. ~~*C. neoformans*~~*CN* can produce melanin in the presence of melanin precursors such as L-dopa. ~~*C.* antarcticus~~In contrast, *CA* is a

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cryptoendolithic inherently- melanized fungus from McMurdo Dry Valleys in Antarctica which grows best at 12-15°C (Selbmann *et al*., 2005).

The most important findings of this study are these:

1) Melanin affords significant protection against high doses (1.5 kGy) of deuterons for both ~~*C. neoformans*~~*CN* and ~~*C. antarcticus*~~*CA*. To the best of our knowledge, this is the first time when the role of melanin in protection of eukaryotic cells against deuterons under physiological conditions (full hydration, temperatures allowing growth) has been reported.

2) Another novel observation of this study is that for both slow growing ~~*C. antarcticus*~~*CA* and fast growing ~~*C. neoformans*~~*CN*, exposure to deuterons resulted in similar metabolic patterns: strong changes in XTT and ATP levels in melanized cells, but not in non-melanized ones. Non-melanized ~~*C. neoformans*~~*CN* and ~~*C. antarcticus*~~*CA* demonstrated different sensitivity to deuterons versus X-rays-: while non-melanized ~~*C. antarcticus*~~*CA* was more resistant to deuterons than non-melanized *C. neoformans*, the latter was more resistant to X-rays.

The explanation possibly lies in the rate of DNA and protein synthesis, which allows the fast growing ~~*C. neoformans*~~*CN* to quickly repair single strand breaks following ~~X ray~~X-rays irradiation-, but cannot help against the double strand DNA breaks induced by deuterons e and in this case the slow growing ~~*C. antarcticus*~~*CA* has the advantage. According to classical radiobiology, rapidly dividing cells tend to be more radiosensitive than slowly dividing ones, for example because they have less time available to repair radiation-induced damage such as DNA double strand breaks before cell dividion ~~are more prone to radiation damage~~ (Hall and Giaccia, 2006). This may explain why slowly-

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growing *CA* was more resistant to radiation than fast-growing *CN*. However, ~~even if the different growth rates play a role in the different resistance observed, some~~ other mechanisms related to the peculiar ecology and adaptations of *CA* could also be involved. ~~This is something that~~These phenomena need~~s~~ to be investigated in the future.

In addition to measuring and modeling cell survival, w~~W~~e used metabolic XTT, MTT and ATP assays in parallel to assess the responses of melanized and non-melanized cells of both species to deuterons and X-rays. Post-~~–~~irradiation interaction at the cell surface between ~~–~~the negatively charged cell-impermeable XTT~~,~~ and melanin, a known electron-shuttle, resulted in elevated XTT levels for both types of radiation and both fungi. The XTT assay may reflect primarily the interaction of radiation with melanin. The same elevation in XTT levels in melanized ~~*C. neoformans*~~*CN* cells was observed in Dadachova *et al.* (2007) when the cells were subjected to gamma radiation with doses thousands of times lower than in this study.

The MTT assay goes deeper into the cell, as MTT is taken into the cells via the plasma membrane potential and is reduced intracellularly. In non-melanized ~~*C. neoformans*~~*CN* cells both deuterons and X-rays ~~caused~~ produced elevation in MTT levels, which is caused by the cells increasing their metabolic rates in response to the radiation insult, perhaps to repair the damage. In melanized ~~*C. neoformans*~~*CN* cells this effect was less pronounced because melanin protects the cells from the damage and may also impede the penetration of MTT into the cell to some extent. In Dadachova *et al.* (2007) no changes in MTT levels were observed post-irradiation in either melanized or non-melanized ~~*C. neoformans*~~*CN* cells, as the radiation doses were very low and non-fungicidal, in contrast to this study.

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As ~~*C. antarcticus*~~*CA* is inherently more resistant to deuterons than ~~*C. neoformans*~~*CN*, its non-melanized cells had barely elevated levels of MTT, possibly because~~as~~ not as much cellular repair was needed as in *CN*. In contrast, since non-melanized ~~*C. antarcticus*~~*CA* is more sensitive to X-rays, its non-melanized cells displayed elevated metabolism and MTT levels required to repair the damage.

Finally, the highly-damaging deuterons caused a decrease in ATP levels in both melanized and non-melanized cells of both fungi, probably reflecting high energy expenditure for damage repair. X-ray exposure did not affect the ATP levels of ~~*C. antarcticus*~~*CA*, probably because changes in ATP production by this very slow growing fungus are below the sensitivity levels of our assay. In melanized ~~*C. neoformans*~~*CN* cells, the X-rays did not produce any changes in ATP levels, and increased ATP in non-melanized ones. This is in contrast to the observations by (Bryan *et al*., 2011) with very low levels of gamma radiation, which decreased the ATP levels in melanized cells and did not change ATP levels in non-melanized cells.

High resistance to different kinds of radiation was reported for dried samples of ~~*C. antarcticus*~~*CA* during preparatory ground-based tests for ESA LIFE EXPOSE-E and BIOMEX EXPOSE-R2 experiments on the ISS (Experiment Verification Tests, EVTs) (Onofri *et al*., 2008; de Vera *et al*., 2014; Pacelli *et al*., 2016). Dried ~~*C. antarcticus*~~*CA* was able to withstand real space vacuum and radiation exposure during the LIFE EXPOSE-E experiment on the ISS (Onofri *et al*., 2012; Onofri *et al*., 2015). Previous studies have found that other organisms that exhibit high resistance to desiccation were also resistant to radiation (Billi *et al*., 2000; Rainey *et al*., 2005; La Duc *et al*., 2007; Daly, 2009) and the survival of ~~*C. antarcticus*~~*CA* after extreme desiccation under 10^{-5} Pa

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vacuum has been reported (Onofri *et al*., 2012). ~~*C. antarcticus*~~*CA* can also survive high doses of UV-B irradiation, with no DNA damage detectable afterwards using PCR approaches (Selbmann *et al*., 2011). The above procedure was set to minimize any possible involvement of the repair system: therefore the remarkable resistance of ~~*C. antarcticus*~~*CA* was explained by its ability to avoid UV-induced DNA damage. Yet, the involvement of the DNA repair system ~~under~~ during/after ionizing radiation exposure under physiological conditions is highly probable as suggested by radiobiological data from other organisms and our modeling results~~the statistical model fit~~. Since the whole genome of ~~*C. antarcticus*~~*CA* was recently sequenced (Sterflinger *et al*., 2014), new insights on the DNA repair potentialities of this fungus will be achieved.

These observations are also of utmost importance for space biological research and planetary protection because the ability to survive ~~at~~ high radiation doses enables microorganisms to potentially contaminate or invade extraterrestrial niches if accidentally transported (Moeller *et al*., 2012). The ability of fungi to withstand harsh outer space conditions and their tendency to contaminate spacecraft has to be taken into consideration because some of them are potential human pathogens capable of endangering the well-being of the astronauts during manned flights. In addition, fungi have strong enzymatic systems and secrete various metabolites, which can degrade structural materials of the spacecraft (Novikova, 2004). The protective role of melanin in fungal cells, coupled with increases in fungal metabolic activity under high energy radiation observed in this study, could open interesting scenarios for searching extraterrestrial life and biosignatures in the Universe (de Vera *et al*., 2012).

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Melanins are ubiquitous in all biological kingdoms, suggesting that they emerged early in the course of evolution, having a double role of protection and possible energy harvesting pigments, when the basic background radiation was higher than today. The same or similar pigments might have enabled life to settle elsewhere in the Universe, exploiting putative extraterrestrial environments, e.g. by using melanin to protect themselves as well as capture and utilize ionizing radiation as an energy source (Dadachova and Casadevall, 2008). It is also worth mentioning that because these fungi produce different types of melanin – DHN melanin by ~~*C. antarcticus*~~*CA* and DOPA melanin by ~~*C. neoformans*~~*CN* – it was impossible to compare the influence of melanin type on radiation protection. This is the subject of further investigation.

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In conclusion, we have demonstrated that melanin pigments present in the fast growing ~~*C. neoformans*~~*CN* and slow growing ~~*C. antarcticus*~~*CA* fungi efficiently protect them against high acute radiation doses. Specifically, our data suggest that melanin is protective not only against sparsely-ionizing photons, but also against deuterons under physiological conditions. These observations are important for basic space radiobiology and for creating melanin-based radioprotectors for humans.

EXPERIMENTAL PROCEDURES

Microorganisms. ~~*Cryomyces antarcticus*~~*CA* CCFEE 515 was isolated by R. Ocampo-Friedmann from sandstone collected at Linnaeus Terrace (Southern Victoria Land) by H. Vishniac, in the Antarctic expedition of 1980-81. It has been supplied by the Culture Collection of Fungi from Extreme Environments (CCFEE), Mycological Section of the Italian Antarctic Museum, (DEB, University of Tuscia, Italy). To obtain non-melanized

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cells of ~~*C. antarcticus*~~*CA*, tricyclazole [5-methyl-1,2,4-triazolo(3,4-b) benzothiazole] was added to cultures for inhibition of the DHN-melanin pathway, at final concentrations of 10 mg/L, according to Cunha, 2005. Melanized and non-melanized ~~*C. antarcticus*~~*CA* were grown on MEA (malt extract agar: malt extract, powdered 30 g/L; peptone 5 g/L; agar 15 g/L; Applichem, GmbH) in Petri dishes for three months. In preparation for the irradiations, 3 days before the irradiation, the ~~*C. antarcticus*~~*CA* colonies were resuspended in physiological solution and then transferred into PBS for the irradiation. American Type Culture Collection (ATCC, Rockville, MD) strain ~~*Cryptococcus neoformans*~~*CN* H99 and its non-melanized laccase mutant deficient Lac(-) (kind gift from Dr. A. Idnurm, Duke University, NC) were used in all experiments. ~~*C. neoformans*~~*CN* was grown in Sabouraud dextrose broth (Difco Laboratories, Detroit, MI) for 24 hrs at 30°C with constant shaking at 150 rpm. Melanized ~~*C. neoformans*~~*CN* cells were generated by growing the fungus in minimal medium with 1 mM 3,4-dihydroxyphenylalanine (L-dopa) for 5–10 days. Before the irradiation, *CN* was transferred into PBS, so the irradiations were performed in PBS for both *CA* and *CN*. After irradiation both strains were grown on their respective solid plates and counted.

Irradiation conditions. Both X-ray and deuteron irradiations were performed at the Radiological Research Accelerator Facility (RARAF), Columbia University, New York. Fungal cell concentration was adjusted to 10^7 per mL (~~*C. antarcticus*~~*CA*) and 10^8 per mL (~~*C. neoformans*~~*CN*) before the irradiation. For X-rays, 1.3 mL of each strain were placed in 1.5 mL Eppendorf tubes for each dose and subjected to 50 kVp ~~x ray~~X-ray irradiation at a dose rate of 5 Gy/min. The deuteron irradiations were performed in customized Mylar-bottom dishes at a dose rate of 1.2-2.5 Gy/sec and at Linear Energy Transfer

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(LET) of 40-~~43-8~~ keV/µm. The doses and the dose rates are described in Table 1. Shortly before irradiation, a small volume (30 µl) of culture medium containing suspended fungal cells was applied to the center of the Mylar surface within the dish, and a glass cover slip was floated on top of the liquid to ensure the formation of a layer with uniform thickness. The dishes (up to 20 at a time) were placed into an aluminum wheel, which was rotated by a computer-controlled motor at a rate defined by the desired dose for each wheel (including zero dose as a control). As the wheel was rotated, a beam of particles sequentially traversed each dish vertically from bottom to top, through the Mylar film and through the overlying cell suspension.

Clonogenic survival assay. Survival of ~~*C. antarcticus*~~*CA* and ~~*C. neoformans*~~*CN* was determined by measuring the number of colony forming units (CFUs) following treatment. After irradiation ~~*C. antarcticus*~~*CA* cells were suspended in 1.3 mL of PBS and diluted to a final concentration of 5,000 cells/mL, and 0.1 mL of the suspension was spread on Petri dishes supplemented with MEA (3 replicates), incubated at 15°C for 3 months and counted. The ~~*C. neoformans*~~*CN* cells were diluted to 4,000 cells/ml, 0.1 ml was plated (three replicates)~~-~~ and the colonies were counted after two to three days.

Mechanistic modeling of clonogenic survival data. The most commonly used mechanistic model for the dose responses of clonogenic survival of irradiated cells is the~~a~~ linear quadratic (LQ) model (Sachs *et al*., 1997; Brenner *et al*., 1998; Hall and Giaccia, 2006). It has been quite successful at describing the data (particularly for mammalian cells) and making robust predictions over a wide range of irradiation scenarios, including dose fractionation or protraction (Brenner *et al*., 1998; Brenner *et al*., 2002). However, there is growing evidence that the inducibility of some DNA repair pathways, and the

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effects of inter- and intra-cellular signaling can alter the shape of the clonogenic survival dose response, particularly at radiation doses where the damage is not lethal to the majority of cells (Bauchwitz and Holloman, 1990; Singh and Krishna, 2006; Coic *et al*., 2008; Xue *et al*., 2015; Fernandez-Palomo *et al*., 2016). The induced repair (IR) model (Marples and Joiner, 1993) which assumes that cells become progressively more radioresistant as radiation dose increases, is a convenient and mechanistically-plausible approach for accounting for the effects of these factors on the survival dose response. Although measurements of DNA damage repair are beyond the scope of the current study, we applied the IR model to the clonogenic survival data for the purpose of quantifying the dose responses for the tested organisms.

We chose the following parametrization for the IR model: linear dose response coefficients for situations when repair and/or pro-survival signaling are fully induced (α_{min}) or not induced (α_{max}), the dose dependence for the induction of repair and/or pro-survival signaling (δ), the quadratic dose response coefficient (β), and the plating efficiency PE under background conditions. The equation for cell surviving fraction S(d) after radiation dose d is:

$$S(d) = PE\, e^{-(\alpha_{min} + (\alpha_{max} - \alpha_{min}) \exp[-\delta\, d])d - \beta\, d^2} \quad (1)$$

At very low doses, when induction of repair has not yet occurred, the term exp[–δ d] remains close to 1 and the dose response predicted by Eq. 1 is dominated by the parameter α_{max}. In contrast, at higher doses repair induction occurs and the term exp[–δ d] becomes close to 0, so the dose response is dominated by the parameter α_{min}. Finally, at very high doses the dose response is dominated by the parameter β. If the inducible

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processes play little/no role in determining survival, then the IR model reduces to the LQ model: $\alpha_{min} = \alpha_{max}$ and $\delta=0$.

Statistical analysis of clonogenic survival data. To analyze the clonogenic survival data, we viewed them as a set of Bernoulli trials, where the number of "successes" is the counted number of colonies on the *i*-th plate ($k(d,i)$) after radiation dose d, and the number of "trials" is the number of plated cells ($n(d,i)$) on the same *i*-th plate. The "success" probability is assumed to have a binomial error distribution (Shuryak *et al.*, 2016).

The log-likelihood function (LL) for this scenario is below, where S(d) is the surviving fraction described by the IR model (Eq. 1):

$$LL = \sum_d \left(\sum_i k(d,i) \ln[S(d)] + \sum_i [n(d,i) - k(d,i)] \ln[1 - S(d)] \right) \quad (2)$$

Here, all constant terms which do not involve S(d) and therefore do not change the results of the optimization were omitted for simplicity.

The LL expression from Eq. 2 is theoretically correct, but the exact values of $n(d,i)$ are not known. Instead, only the average values for the numbers of seeded cells N(d) are available. Consequently, we modified Eq. 2 as follows, to produce LL_e which is the LL expression using only the data that are available:

$$LL_e = \sum_d \left(\sum_i k(d,i) \ln[S(d)] + \sum_i max[0,\ N(d) - k(d,i)] \ln[1 - S(d)] \right) \quad (3)$$

Here, the expression $max(0, N(d) - k(d,i))$ is used to account for the instances when $k(d,i)$ could, by chance, become > N(d). Such instances would be rare for radiation doses substantially larger than zero, where S(d) would be << 1.

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To implement this data analysis approach, we determined the best-fit values of parameters which enter into the model for S(*d*), for which the LL_e function (Eq. 3) was maximized. The maximization was performed using the sequential quadratic programming (SQP) algorithm in Maple 2015® software. The plating efficiency (PE), which is unrelated to radiation, was allowed to be different for different experimental dates, whereas all four radiation dose response parameters (α_{min}, α_{max}, δ, β) were kept constant for all experiments using the given fungal strain and radiation type. The parameter values were restricted to ≥ 0 to maintain mechanistic plausibility.

The probability of finding the global maximum (rather than local maxima) was enhanced by using 100 random initial conditions for the model parameters. Uncertainties (95% CIs) for best-fit model parameter values were estimated by profile likelihood as follows: 10,000 Monte-Carlo-generated parameter values in the vicinity of the best-fit values were used to estimate the critical contour of the log likelihood function, which is based on the asymptotic X^2 behavior of the log likelihood distribution.

To quantify the effect of melanization status on the clonogenic survival of each tested species, we compared the plating efficiencies for melanized and non-melanized strains at the same radiation dose. The observed plating efficiency is the ratio of the number of counted colonies to the number of plated cells. Uncertainties (95% CIs) for the plating efficiency were calculated by the score confidence interval approach for binomial proportions (Agresti and Coull, 1998). Ratios of plating efficiencies for melanized vs non-melanized strains and corresponding p-values were estimated by Monte Carlo simulation.

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Determination of metabolic activity of melanized and non-melanized cells subjected to deuteron and X-rays radiation by XTT and MTT assays. After irradiation, melanized and non-melanized fungal cells were washed, suspended in PBS and 100 µL cells at 10^5 - 10^6 cells/ml concentration were placed into wells in 96 well plates, 3 wells for each condition. For XTT (2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide) assay 40 µL (1 mg/mL XTT in PBS) and 4 µL 1mM menadione in acetone were added to each well, the plates were covered with foil and incubated at room temperature for 2, 3, 4 and 12 hours. We show the readings obtained after 4 hours. The absorbance (Labsystem Multiskan, Franklin, MA) was read at 492 and 650 nm, and the absorbance at 650 nm was subtracted from the absorbance at 492 nm. For MTT (2-(4,5-dimethyl-2-thiazolyl)-3,5-diphenyl-2H-tetrazolium bromide) assay, an MTT solution in PBS was added to the wells with the cells, so that the final MTT concentration was 0.5 mg/mL. After incubation at room temperature for 24 hours and 72 hours, the cells were spun down at 2,000 rpm, supernatant was discarded, followed by addition of 100 µL 10% Sodium dodecyl sulfate (SDS) in 50% dimethylformamide (DMF) to each well. The absorbance was read at 550 and 650 nm, and the absorbance at 650 nm was subtracted from the absorbance at 550 nm. The results show the 24 hours readings. Both XTT and MTT results were normalized by the number of cells per well and these values were again normalized by dividing by the control values which were the non-irradiated cells at time zero.

ATP assay. Following experimental treatments, the cellular extracts were prepared for adenosine triphosphate (ATP) measurements by freezing 0.2 mL cells in 0.5 mL tubes in a dry ice-ethanol bath. TE buffer (0.1 mL) was added to the frozen cells and the

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mixture was boiled for two minutes. Cell debris was pelleted by centrifugation at 1000 rcf for 10 min, and 10 µL/well of the cell supernatants were assayed for ATP using the luciferase/luciferin kit from Molecular Probes (Eugene, Oregon, USA). Emitted light was measured in a Wallac Perkin Elmer Victor 2 luminometer/plate reader (Waltham, MA, USA). Measurements were made in triplicate for each treatment. The assay was linear over a range of extracts derived from 0.3 to 2.6 x 10^5 cells. The luminometer readings in the luciferase assay increased for about 10 min after addition of the substrate and enzymes, followed by a gradual decrease in luminescence. Readings recorded at 5 min are shown. ATP concentrations were calculated using a standard curve for every assay. The ATP concentrations were then normalized as for XTT and MTT assays, first by dividing the ATP concentration by cell number, and then dividing that value by the control value which were the non-irradiated cells at time zero.

Statistical analysis and descriptive modeling of metabolic assay data. To the best of our knowledge, mechanistic modeling of metabolic assay data after irradiation is not as developed as modeling of clonogenic survival. Consequently, we used simple descriptive modeling (multiple linear regression) on the metabolic assay data. The response variable (either XTT, MTT or ATP) measured in each sample was normalized by the mean for non-irradiated control samples, and logarithm-transformed. The transformation was intended to bring the error distribution closer to the normal distribution, and to avoid biologically impossible negative values. The independent variables were radiation dose and dose squared. The linear regression was performed separately for each fungal strain and radiation type.

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TABLES

Table 1. Deuterons and X-rays radiation doses used in the study.

Deuterons	Applied doses (Gy)	X-rays	Applied doses (Gy)
LET: 40-43 KeV/um Dose Rate: 1.2-2.5 Gy/sec	0	Dose Rate: 5 Gy/min	0
	50		
	250		150
	500		
	1500		300

Table 2. Comparison of clonogenic survival of melanized vs non-melanized strains of *CN* and *CA* after the highest tested doses of deuteron and X-rays irradiation. For each replicate experiment, the ratio of plating efficiencies (and its 95% CIs) for melanized vs non-melanized cells represents the ratio of clonogenic survival: values >1 indicate that melanized cells had higher survival than their non-melanized counterparts. The p-value represents the probability that the plating efficiency ratio was ≤1.

Organism	Radiation type and dose	Replicate experiment	Plating efficiency ratio: melanized vs non-melanized			p-value
			value	95% CIs		
CN	deuterons, 1.5 kGy	A	10.75	5.72	29.62	$<10^{-4}$
		B	4.50	2.12	15.72	$<10^{-4}$
CA	deuterons, 1.5 kGy	A	1.73	1.59	1.89	$<10^{-4}$
CN	X-rays, 0.3 kGy	A	2.54	2.12	3.04	$<10^{-4}$
		B	1.11	0.86	1.42	0.212
		C	1.16	0.92	1.45	0.106
CA	X-rays, 0.3 kGy	A	87.94	65.33	131.35	$<10^{-4}$
		B	4.94	4.37	5.62	$<10^{-4}$

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Table 3. Best-fit model parameters for clonogenic survival of irradiated *CN* and *CA*. Italic font indicates parameter values which are significantly affected by melanization status. Parameter meanings are as follows: linear dose response coefficients for situations when DNA repair and/or pro-survival signaling are fully induced (α_{min}) or not induced (α_{max}), the dose dependence for the induction of DNA repair and/or pro-survival signaling (δ), the quadratic dose response coefficient (β).

Organism	Radiation type	Mela-niza-tion	Best-fit model parameters											
			α_{min} (kGy^{-1})			α_{max} (kGy^{-1})			δ (kGy^{-1})			β (kGy^{-2})		
			Value	95% CIs		value	95% CIs		value	95% CIs		value	95% CIs	
CN	Deuterons	Yes	0.00	0.00	0.00	*13.84*	*12.9*	*15.06*	*3.58*	*3.37*	*4.56*	1.24	0.62	1.33
		No	0.00	0.00	0.00	*5.54*	*5.07*	*6.04*	*1.00*	*0.63*	*1.62*	1.20	0.60	1.62
	X-rays	Yes	1.28	0.64	2.56	8.06	4.23	16.07	8.36	4.19	16.68	1.95	0.98	3.89
		No	0.71	0.14	3.57	2.75	0.55	13.79	7.99	1.60	40.06	7.72	1.54	11.47
CA	Deuterons	Yes	0.00	0.00	0.00	*1.99*	*1.85*	*2.14*	1.10	0.86	1.68	0.27	0.03	0.34
		No	0.00	0.00	0.00	*3.70*	*3.57*	*3.86*	0.80	0.76	0.94	0.00	0.00	0.04
	X-rays	Yes	0.40	0.04	3.99	*0.94*	*0.09*	*9.44*	2.12	0.21	21.22	5.13	0.51	7.71
		No	1.64	0.26	9.24	*10.96*	*10.0*	*69.12*	1.03	0.32	6.52	1.42	0.23	8.96

Supplementary Table

Table 1S. Best-fit regression parameters for metabolic responses of irradiated *CN* and *CA*. Italic font indicates parameter values which are significantly affected by melanization status, and bold font indicates values which are significantly different from zero (significance was defined by p-values < 0.05). The linear dose response parameter dominates the response at low doses, and the quadratic parameter dominates the response as high doses.

Organism	Assay type	Radiation type	Mela-nization	Dose response parameters for ln normalized XTT, MTT or ATP						Fraction of variance explained (R^2)
				linear (kGy^{-1})			quadratic (kGy^{-2})			
				value	95% CIs		value	95% CIs		
CN	XTT	Deuterons	yes	***0.82***	***0.60***	***1.04***	***-0.26***	***-0.43***	***-0.10***	0.73
			no	***-0.64***	***-0.94***	***-0.34***	***0.61***	***0.39***	***0.83***	0.35
		X-rays	yes	-0.40	-1.90	1.10	5.80	0.38	11.22	0.70
			no	-0.25	-1.54	1.04	1.56	-3.10	6.22	0.06
	MTT	Deuterons	yes	0.41	0.04	0.79	-0.07	-0.33	0.19	0.70
			no	0.40	-0.02	0.82	0.28	-0.02	0.57	0.92
		X-rays	yes	0.21	-1.83	2.24	0.13	-7.24	7.50	0.08
			no	-0.23	-2.47	2.01	4.33	-3.76	12.43	0.53
	ATP	Deuterons	yes	***-2.32***	***-2.63***	***-2.01***	***1.06***	***0.83***	***1.28***	0.92
			no	***-0.82***	***-0.95***	***-0.70***	***0.19***	***0.10***	***0.28***	0.96

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		X-rays	yes	***0.61***	***0.27***	***0.95***	***-1.28***	***-2.51***	***-0.05***	0.80
			no	*-0.65*	*-1.36*	*0.06*	***6.28***	***3.71***	***8.86***	0.94
CA	XTT	Deuterons	yes	***0.78***	***0.59***	***0.97***	***-0.43***	***-0.56***	***-0.30***	0.65
			no	*0.07*	*-0.08*	*0.23*	*-0.04*	*-0.15*	*0.06*	0.02
		X-rays	yes	1.47	0.62	2.31	-2.20	-5.25	0.86	0.74
			no	0.66	-0.28	1.61	-1.69	-5.10	1.72	0.13
	MTT	Deuterons	yes	-0.19	-0.43	0.05	0.15	-0.01	0.32	0.07
			no	0.21	0.04	0.37	-0.10	-0.21	0.02	0.26
		X-rays	yes	-1.01	-2.67	0.65	2.10	-3.89	8.08	0.22
			no	0.46	-0.97	1.89	0.60	-4.57	5.78	0.41
	ATP	deuterons	yes	-0.21	-0.29	-0.13	0.04	-0.01	0.10	0.83
			no	-0.03	-0.15	0.08	-0.02	-0.10	0.06	0.25
		X-rays	yes	-0.09	-0.40	0.22	-0.10	-1.22	1.03	0.32
			no	0.00	-0.32	0.32	0.15	-1.00	1.30	0.07

FIGURE LEGENDS

Fig. 1. Clonogenic survival of melanized and non-melanized *CN* and *CA* after deuteron irradiation: **A)** *CN*; **B)** *CA*. Symbols represent data and curves represent IR model fits. Several symbols at the same radiation dose and fungal strain represent data from different replicate experiments. Error bars represent 95% CIs. Blue = melanized, red = non-melanized; **C)** Comparison of clonogenic survival of melanized vs non-melanized strains of *CN* (black) and *CA* (green) after the highest tested doses of deuterons and X-ray irradiation. Error bars represent 95% CIs.

Fig. 2. Clonogenic survival of melanized and non-melanized *CN* and *CA* after X-ray irradiation: **A)** *CN*; **B)** *CA*. Symbols represent data and curves represent IR model fits. Error bars represent 95% CIs. Blue = melanized, red = non-melanized; **C)** Comparison of clonogenic survival of melanized vs non-melanized strains of *CN* (black) and *CA* (green) after the highest tested doses of deuterons and X-ray irradiation. Error bars represent 95% CIs.

Fig. 3. Effects of irradiation on *CN* XTT reducing activity. **A)** Melanized cells exposed to deuterons. **B)** non-melanized cells exposed to deuterons. **C)** melanized cells exposed to X-rays. **D)** non-melanized cells exposed to X-rays. Closed symbols represent melanized cells, and open symbols represent non-melanized cells. The results were normalized by the number of cells per well and these values were again normalized by dividing by the control values which were the non-irradiated cells at time zero, n stands for normalized.

Fig.4. Effects of irradiation on *CA* XTT reducing activity. **A)** Melanized cells exposed to deuterons. **B)** non-melanized cells exposed to deuterons. **C)** melanized cells exposed to X-rays. **D)** non-melanized cells exposed to X-rays. Closed symbols represent melanized cells, and open

symbols represent non-melanized cells. The results were normalized by the number of cells per well and these values were again normalized by dividing by the control values which were the non-irradiated cells at time zero, n stands for normalized.

Fig. 5. Effects of irradiation on *CN* MTT reducing activity. **A)** Melanized cells exposed to deuterons. **B)** non-melanized cells exposed to deuterons. **C)** melanized cells exposed to X-rays. **D)** non-melanized cells exposed to X-rays. Closed symbols represent melanized cells, and open symbols represent non-melanized cells. The results were normalized by the number of cells per well and these values were again normalized by dividing by the control values which were the non-irradiated cells at time zero, n stands for normalized.

Fig. 6. Effects of irradiation on *CA* MTT reducing activity. **A)** Melanized cells exposed to deuterons. **B)** non-melanized cells exposed to deuterons. **C)** melanized cells exposed to X-rays. **D)** non-melanized cells exposed to X-rays. Closed symbols represent melanized cells, and open symbols represent non-melanized cells. The results were normalized by the number of cells per well and these values were again normalized by dividing by the control values which were the non-irradiated cells at time zero, n stands for normalized.

Fig. 7. Effects of irradiation on *CN* ATP level. **A)** Melanized cells exposed to deuterons. **B)** non-melanized cells exposed to deuterons. **C)** melanized cells exposed to X-rays. **D)** non-melanized cells exposed to X-rays. Closed symbols represent melanized cells, and open symbols represent non-melanized cells. The results were normalized by the number of cells per well and these values

were again normalized by dividing by the control values which were the non-irradiated cells at time zero, n stands for normalized.

Fig. 8. Effects of irradiation on *CA* ATP level. **A)** Melanized cells exposed to deuterons. **B)** non-melanized cells exposed to deuterons. **C)** melanized cells exposed to X-rays. **D)** non-melanized cells exposed to X-rays. Closed symbols represent melanized cells, and open symbols represent non-melanized cells. The results were normalized by the number of cells per well and these values were again normalized by dividing by the control values which were the non-irradiated cells at time zero, n stands for normalized.