Rapamycin as a potential treatment for succinate dehydrogenase deficiency

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Abstract

\textit{Drosophila melanogaster} is a powerful model to study mitochondrial respiratory chain defects, particularly succinate dehydrogenase (SDH) deficiency. Mutations in \textit{sdh} genes cause degenerative disorders and often lead to death. Therapies for such pathologies are based on a combination of vitamins and dietary supplements, and are rarely effective. In \textit{Drosophila}, mutations in several of the genes encoding SDH resemble the pathology of SDH deficiency in humans, enabling the \textit{Drosophila} model to be used in finding treatments for this condition. Here we show that exposure to the drug rapamycin improves the survival of \textit{sdh} mutant strains, the activity of SDH and the impaired climbing associated with \textit{sdh} mutations. However, the production of reactive oxygen species, the oxygen consumption of isolated mitochondria and the resistance to hyperoxia were minimally affected. Our results contribute to the current research seeking a treatment for mitochondrial disease.

Keywords: Genetics, Biochemistry, Cell biology, Physiology
1. Introduction

Defects in mitochondrial succinate dehydrogenase (SDH), or complex II of the electron transport chain are a rare cause of mitochondrial disease. Despite this rarity, they are associated with a broad range of disorders and a variety of clinical presentations [1, 2, 3, 4]. SDH is composed of four subunits (A, B, C and D), which, in contrast with other respiratory chain complexes, are nucleus-encoded and inherited in an autosomal fashion. Mutations in \textit{sdhA}, which encodes for the subunit A of SDH, as well as mutations in SDHAF1 (succinate dehydrogenase assembly factor 1) are rare causes of Leigh syndrome. Leigh syndrome is the most common pediatric representation of mitochondrial disease and it is genetically heterogeneous, however patients with Leigh syndrome are more likely to have mutations in complex I and complex IV of the electron transport chain [5]. Leigh syndrome is characterized by an early onset of progressive neurodegeneration [6, 7] marked by developmental delay, weakness, ataxia, dystonia, lactic acidosis and ophthalmoplegia, seizures [3, 5].

Mutations in the genes encoding the SDH and SDHAF2 (succinate dehydrogenase assembly factory 2) have been associated with tumor formation, especially paragangliomas (PGLs), in addition to pheochromocytomas, renal cell carcinomas, gastrointestinal stromal tumors, pituitary adenomas, thyroid cancer and neuroblastomas [8, 9, 10, 11]. Anti-cancer drugs that target SDH have shown promise in treating specific types of tumors in animal and cellular models [12], but the efficacy of treatments for SDH deficiency depend on the marked phenotypic heterogeneity of the disease [1]. The striking differences observed among phenotypes associated with SDH deficiency might originate from SDH’s position at the intersection of key pathways in energy production: the citric acid cycle and the electron transport chain. SDH performs this dual role located in the inner mitochondrial membrane where it oxidizes succinate into fumarate in the citric acid cycle and it reduces ubiquinone in the process of oxidative phosphorylation as complex II of the electron transport chain [1, 4, 11, 13]. Therefore, defects in its operation will affect the homeostatic nature of metabolic networks and a complex organelle-systemic response [14].

Overall, therapies for mitochondrial disorders are normally based on vitamin supplements, modifications to diet and exercise [15, 16, 17]. While there are no FDA-approved pharmaceuticals that specifically target mitochondrial disorders [18], multiple drug treatments are currently under study to evaluate their potential as therapies for genetic mitochondrial disorders. Some treatments are promising, such as para-benzoquinone EPI-743, which was shown to arrest neuromuscular degeneration in Leigh syndrome patients, however, no drug have offered a reliable treatment yet [19, 20].
One such emerging potential treatment for mitochondrial disorders is rapamycin [17, 21], an immunosuppressant drug currently approved by the FDA for preventing rejection in transplant and stent patients [22]. It operates by inhibiting the mTOR (mechanistic Target of Rapamycin) nutrient signaling pathway, slowing protein translation, protein transcription and metabolic rate, while modulating cellular growth, metabolism, and apoptosis [23, 24]. Following studies demonstrating rapamycin’s potential to affect tumor growth, metabolic disorders such as diabetes, and neurodegenerative disorders, interest in rapamycin and its analogues has skyrocketed [23, 25, 26, 27]. Research in the mouse and the fly model, for example, demonstrated that rapamycin alleviates the pathology of complex I deficiency; however, the mechanism by which it achieves this effect in the mitochondria remains unknown [21, 28].

In D. melanogaster we previously described that rapamycin improves mitochondrial function, increases SDH enzymatic activity, and decreases the production of reactive oxygen species (ROS) [29]. Because these effects may be beneficial for patients with SDH deficiency, we elected to further study rapamycin as a potential treatment for SDH deficiency using Drosophila as our genetic model. Within this model system, multiple SDH deficient mutant strains have been discovered to display encephalopathy, neurological degeneration, metabolic dysfunction, and reduced lifespan, closely mimicking the complications of SDH deficiency in humans [30, 31, 32] and creating a model for Leigh syndrome. Additionally, a clear link between aging and the production of ROS has been found both in wild type strains and in mitochondrial mutant strains [33]; in particular, mutations in the sdhB gene, which encodes the iron-sulfur binding subunit of the SDH enzyme, have been found to cause decreased longevity, increased ROS production, and an overall reduction in health in flies, as measured by their climbing ability [32]. Mutations in the sdhA gene have comparable neurological consequences to those described in Leigh syndrome patients, with sdhA mutant retina cells suffering from the degeneration of their synapses and cell bodies [31] as a result of increased levels of ROS production. Pharmacological and genetic manipulations that reduce ROS levels prevents synapse degradation [31]. Since one of the most critical limitations in understanding the role of SDH deficiency in diseases is the limitation of cell and animals models [4], studies in Drosophila melanogaster are important to understand the mechanisms underlying mitochondrial respiratory chain defects and find potential treatments.

2. Results

2.1. Rapamycin improved the climbing ability and SDH activity in sdhB mutants

Previous climbing assays have confirmed the reduced physical ability of flies with the sdhB mutant allele sdhB<sup>ey12081</sup> when compared to the control sdhB<sup>ex29</sup> flies.
Exposure to rapamycin exhibited increased climbing ability in both *sdhB*\textsuperscript{ey12081} mutant and *sdhB*\textsuperscript{ex29} control strain (Fig. 1A). Since rapamycin has been also shown to increase SDH activity and the oxygen consumption of *wild type* flies [29], we investigated its potential effects on the activity of SDH and on the respiration of isolated mitochondria from the *sdhB* mutant flies. 200 μM rapamycin treatment significantly increased succinate dehydrogenase activity after two days of treatment in *sdhB*\textsuperscript{ey12081} and in *sdhB*\textsuperscript{ex29} flies (Fig. 1B). Similarly to what we previously described in *wild type* flies [29], mitochondria isolated from *sdhB*\textsuperscript{ex29} control flies treated with rapamycin showed an increase in oxygen consumption during ADP dependent respiration (state 3). However, the oxygen consumption of isolated mitochondria treated with rapamycin was not increased in *sdhB*\textsuperscript{ey12081} mutant flies (Fig. 1C).

2.2. Rapamycin increased the longevity of *SdhB* mutants

As described previously, 200μM rapamycin treatment extended the longevity of *wild type* flies [34] (Fig. 2A and B). Flies deficient for the subunit B of SDH, *sdhB*\textsuperscript{ey12081} mutants, exhibited reduced longevity when compared to control flies [32] (Fig. 2B). This reduced longevity was partially rescued when flies were exposed to rapamycin treatment (Fig. 2B).

![Fig. 1. Effects of rapamycin on the climbing, SDH activity and mitochondrial respiration of *sdhB* mutants.](https://doi.org/10.1016/j.heliyon.2019.e01217)
2.3. Reduction in lifespan caused by hyperoxia is minimally affected by rapamycin

High levels of oxygen (hyperoxia) cause flies to die prematurely from oxidative stress, with sdhB mutants displaying significantly reduced lifespans in comparison to the control [32] (Fig. 2C). Exposure to rapamycin significantly, but minimally, increased the longevity of sdhB<sup>Δ12081</sup> mutant flies under hyperoxic conditions (Fig. 2C). Rapamycin, however, did not affect the longevity of sdhB<sup>Δ29</sup> control flies (Fig. 2C).

Fig. 2. Effects of rapamycin on the longevity under normoxia and hyperoxia and ROS production (H<sub>2</sub>O<sub>2</sub>) on SdhB mutants. (A) Longevity analysis of increasing concentration of rapamycin on wild type flies. Significance was determined by Log rank test [0–5 μM p-value: 0.7; 0–50 μM p-value: 0.25; 0–100 μM p-value: 0.23; 0–200 μM p-value: 0.01, 0–400 μM p-value: 0.9]. (B) Longevity analysis of SdhB<sup>Δ12081</sup> (red) and sdhB<sup>Δ29</sup> (black) strains exposed to 200 μM of rapamycin (dotted) and vehicle control (filled symbols) under normal oxygen conditions. Significance was determined by Log rank test [SdhB<sup>Δ29</sup> control-rapamycin p-value: 0.0001; SdhB<sup>Δ12081</sup> control-rapamycin p-value: 0.0001]. (C) Longevity analysis of SdhB<sup>Δ12081</sup> (red) and sdhB<sup>Δ29</sup> (black) strains exposed to 200 μM of rapamycin (dotted) and vehicle control (filled symbols) under hyperoxia. Significance was determined by Log rank test [SdhB<sup>Δ29</sup> control-rapamycin p-value: 0.17; SdhB<sup>Δ12081</sup> control-rapamycin p-value: 0.021. (D) H<sub>2</sub>O<sub>2</sub> production of isolated mitochondria from sdhB<sup>Δ12081</sup> and sdhB<sup>Δ29</sup> female flies after two days of 200 μM rapamycin treatment (white) and control (grey). Significance was determined by ANOVA were velocity of the production of H<sub>2</sub>O<sub>2</sub> (in mOD/min/mg/ml(protein)) is the dependent variable, and the genotype and treatment are fixed values. Treatment [F: 6.10; p-value: 0.04], genotype [F: 28.2; p-value: 0.0001], treatment*genotype [F: 0.104; p-value: 0.752]. *p-value < 0.05.
2.4. Rapamycin did not decrease the elevated production of ROS of SdhB mutants

Because of our previous work with isolated wild type mitochondria, where we showed that rapamycin treatment generates less hydrogen peroxide, a form of ROS [29], levels of hydrogen peroxide were measured in sdhB<sup>ye12081</sup> and sdhB<sup>ex29</sup> flies after rapamycin administration (Fig. 2D). The production of hydrogen peroxide was increased in sdhB<sup>ye12081</sup> mutant flies compared to sdhB<sup>ex29</sup> control flies (Fig. 2D). Rapamycin treatment minimally decreased the production of hydrogen peroxide in the sdhB mutant strain (Fig. 2D).

2.5. Rapamycin improved the survival of sdhA mutants

sdhA mutant flies die during first instar larvae [31]. It has been suggested that the premature death is caused by elevated levels of ROS since flies with retina cells that are homozygous for sdhA mutant alleles have reduced neuronal degeneration after treatment with the well-known antioxidant vitamin E [31]. To study the effect of increasing concentration of rapamycin and vitamin E on the survival of sdhA mutant larvae, sdhA<sup>1404</sup> flies were exposed to increasing concentrations of rapamycin (from 0 to 10 μM) and vitamin E (from 0 to 250 mg/ml) (Fig. 3A and B). Vitamin E treatment did not affect the survival of sdhA<sup>1404</sup> larvae (Fig. 3B), however, 7 and 10 μM rapamycin double the maximal survival of sdhA homozygous mutant larvae (Fig. 3A). We confirmed this result using an independently created sdhA mutant allele: sdhA<sup>1</sup>. In both alleles of sdhA, larvae in the presence of rapamycin lived up to ten days, while control larvae died around day five (Fig. 3C). All flies died before reaching pupation.

Although our experiment with vitamin E suggested that the mechanism as to how rapamycin may extend larval survival of sdhA mutant is independent of its function as antioxidant, we studied the effect of rapamycin on ROS production during larvae (Fig. 3D). We exposed sdhA<sup>1404</sup> heterozygous larvae to 10 μM of rapamycin and measured the production of H<sub>2</sub>O<sub>2</sub> during third instar larvae where abundant mitochondrial enriched fraction could be obtained unlike samples from homozygous sdhA individuals since the later are very scarce and they die before reaching second instar larvae. Rapamycin did not decrease the production of H<sub>2</sub>O<sub>2</sub> during third instar larvae (Fig. 3D) suggesting that the increase in survival of sdhA<sup>1404</sup> by rapamycin is independent of an antioxidant effect.

3. Discussion

The diagnosis of the multifaceted pathology associated with mitochondrial disorders, despite their relative rarity, has improved in the recent years. The complex nature of metabolic pathways, along with the intertwined genetic and environmental interactions that influence them, are among the most prominent reasons as to why
mitochondrial treatments are only partially effective and vary from patient to patient [16]. Additionally, the lack of model systems has also injured efforts in understanding and developing new treatments, especially for SDH deficiency [4]. In this work, *Drosophila* was used as a model system to find a treatment for SDH deficiency. The obtained results indicate that the drug rapamycin benefits the pathology associated with succinate dehydrogenase deficiency, improving the climbing and the enzymatic activity of SDH (Fig. 1) in *sdhB* homozygous mutants flies and increasing the survival of *sdhA* and *B* homozygous mutants individuals (Figs. 2 and 3). The molecular mechanism as to how rapamycin is benefiting SDH deficiency does not seem to be dependent on its modulation of mitochondrial respiration, since in respiration of the mitochondria isolated from rapamycin treated *sdhB* mutant flies was not affected (Fig. 1C). The data presented here suggest that in both, *sdhB* and *sdhA* mutant

![Graph A](image1.png)  
**A** Survival of *sdhA* exposed to increasing concentration of rapamycin (A) and vitamin E (B). Significance was determined by Log rank test [0—3 μM rapamycin p-value: 0.307; 0—7 μM rapamycin p-value: 0.033, 0—10 μM rapamycin p-value: 0.004; 0—50 mg/ml vitamin E p-value: 0.447, 0—100 mg/ml vitamin E p-value: 0.57; 0—200 mg/ml vitamin E p-value: 0.292; 0—250 mg/ml vitamin E p-value: 0.66]. (C) Survival of *sdhA* (black) and *sdhA* (red) mutant larvae exposed to 10 μM of rapamycin (dotted) and vehicle control (filled). Significance was determined using Log rank test [sdhA* control-rapamycin p-value: 0.003; *sdhA* control-rapamycin p-value: 0.001]. (D) H2O2 production in mOD/min/mg/ml (protein) of isolated mitochondria from heterozygous *sdhA* third instar larvae exposed to 10 μM rapamycin (white) since embryo. Significance was determined by independent student t-test, [p-value: 0.450]. * p-value < 0.05.
alleles, rapamycin antioxidant capacity may not be the main cause for the improvement of the pathology of SDH deficient flies, since levels of ROS were minimally decreased in sdhB mutant flies (Fig. 2D) or sdhA heterozygous larvae (Fig. 3D). This goes along with our results showing that the survival of homozygous sdhA mutant larvae was not improved after treatment with vitamin E.

Overall, our research presents rapamycin as a potential treatment for SDH deficiency; however our work fails to provide a mechanism as to how rapamycin is doing so. We have previously reported that the beneficial effects of rapamycin on mitochondrial physiology requires changes in mitochondrial metabolism as evidenced by the shift on mitochondrial metabolites after rapamycin treatment [29]. Complementary to our research, rapamycin has been shown to improve the pathology of complex I deficiency in a way that seems to affect metabolism and that is independent of autophagy [21]. Previous research combined with results presented here, suggest that the beneficial effects of rapamycin observed in sdhB and sdhA mutants may be mediated by an increase in both available energy and metabolic efficiency, as indicated by the rise in SDH enzymatic activity (Fig. 1B), rather than an increase in oxygen consumption (Fig. 1C) or a decrease in ROS production (Figs. 2D and 3D). However, the mechanism as to how rapamycin is improving SDH deficiency is still unknown. Yet our data combined with our previous work suggest that rapamycin acts upstream of oxidative phosphorylation.

Regardless of considerable research elucidating rapamycin’s mechanisms of action, many of the drug’s effects on different protein networks downstream of TORC1 and 2 remain unknown. Since both TORC1 and TORC2 sit at the node of large, complex, highly integrated nutrient signaling networks, perturbation of those pathways with rapamycin produces the strong but not unilaterally beneficial responses in organismal and molecular processes and may depend on the duration of the treatment and its function as dietary restriction mimetic [35, 36, 37]. In this study we present the first assessment of the effects of rapamycin on SDH deficiency in Drosophila. Our research contributes to the growing body of data supporting TOR suppression’s potential as a preliminary treatment for Leigh’s syndrome and other mitochondrial disorders [21, 28, 38, 39]. This disease represents a serious threat to the health of a small but growing population of patients who find themselves pharmaceutically neglected. Research into rapamycin’s potential as a treatment to alleviate mitochondrial disorders may help to promote the health and well-being of patients suffering from these devastating diseases.

4. Methods

4.1. Fly strains

Strains of D. melanogaster were obtained courtesy of Dr. David Walker, Molecular Biology Institute, UCLA, CA and are described in [32]. In brief, the sdhB

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mutation studied is a transposable element insertion line, first described by the Berkeley *Drosophila* Genome Project as a P-element transposon containing *white* and *yellow* inserted into the 5′ untranslated region of the *sdhB* nuclear gene on chromosome 2R. Subsequent precise excision of the p-element produced the *sdhBex29* control strain [32]. *sdhA* mutant strains were *sdhA*1404, with Valine-445 changed to Glutamate and *sdhA*1 an natural occurring mutation with unknown lesion. Both were obtained courtesy of Dr. Thomas Clandinin, Stanford University, and are discussed in [31].

All fly stocks were maintained in normal media (11% sugar, 2% autolyzed yeast, 5.2% cornmeal, agar 0.79% w/v in water, and 0.2% tegosept/methyl p-hydroxybenzoate (Spectrum Chemical, Gardena, CA) and kept at 25 °C and 60% humidity in 12-hour cycles of light and darkness. Prior to placement on experimental treatment, flies were density controlled for 1 generation by 5-day egg lays by either 25 pairs of parents in bottles containing 50 mL food or 5 pairs of parents in vials containing 5.0 mL food.

4.2. Rapamycin treatment

Rapamycin was obtained commercially from LC Laboratories, Woburn, MA. It was dissolved in ethanol and added to media containing at final concentration of rapamycin as specified on the figures. For this study we used female flies since our previous research describing the effects of rapamycin on mitochondrial physiology were done in females [29].

4.3. Climbing assay

Because *Drosophila* exhibit a negative geotaxis reflex, or the response to climb upward against gravity, negative geotaxis assays are frequently used to assess fly health and behavior [32]. Adult female flies 0–4 days after eclosion were collected and placed on media with either rapamycin or ethanol vehicle control, with ten flies per 25 × 95 mm vial containing 5 mL food. Flies were maintained on treatment or control for two days, then removed and placed into empty vials measuring 25 × 95 mm capped with cotton. Following an acclimation period of five minutes, each vial was tapped gently to force all flies to the bottom, after which the number of flies out of the total that were able to climb upwards at least 65 mm was recorded. Statistical analysis was performed using an Analysis of Variance (ANOVA) in IBM SPSS Statistics 21 as specified in the figure legends.

4.4. SDH activity

After two days of rapamycin treatment, flies were removed and homogenized in 1 mL of chilled isolation buffer (225 mM mannitol, 75 mM sucrose, 10 mM
MOPS, 1 mM EGTA, 0.5% BSA at pH 7.2). The whole-fly lysate was then centrifuged at 300 rcf for 5 minutes at 4 °C. The resulting supernatant was then centrifuged again at 6,000 rcf for 10 minutes at 4 °C. The pellet obtained was then resuspended in 100 μL of respiration buffer (225 mM mannitol, 75 mM sucrose, 10 mM KCl, 10 mM Tris-HCl, 5 mM KH2PO4, pH 7.2) over ice and considered the mitochondrial suspension [40]. The SDH activity was measured as described in [29]. Enzymatic activity was normalized by protein concentration, measured by Bradford reagent (Sigma-Aldrich, St. Louis, MO) following the manufacturer’s protocol. The velocity of the reaction is represented as the change in absorbance per minute per protein concentration: mOD/min/mg/ml. The significance was determined using Analysis of Variance (ANOVA) in IBM SPSS Statistics 21 as specified in the figure legends.

4.5. H2O2 production

Flies were exposed for two days of rapamycin treatment for adult sdhB mutant flies, and since embryo to until third instar larvae stage for sdhA mutant flies. After rapamycin exposure flies were removed and a mitochondrial suspension was prepared in the same manner as described above for the SDH enzymatic assay. This suspension was then assayed for H2O2 production using an Amplex Red/horseradish peroxidase assay kit (Invitrogen, Carlsbad, CA) following the manufacturer’s specifications. The production of H2O2 was normalized by protein concentration, measured by Bradford reagent (Sigma-Aldrich, St. Louis, MO) following the manufacturer’s protocol. Data represents the velocity of the production of H2O2 as the change in absorbance per minute per protein concentration: mOD/min/mg/ml. The significance was determined using Analysis of Variance (ANOVA) for Fig. 1 or using a student t-test Fig. 3 in IBM SPSS Statistics 21 as specified in the figure legends.

4.6. Demographies

Female flies density-controlled for one generation were collected 0–4 days following eclosion and placed in 1 liter PET cylindrical cages measuring 8.0 cm diameter at the base, 11.5 cm diameter at the top, and 14.5 cm in height. 50–100 flies were placed in a single demography cage and fed either rapamycin treatment or vehicle control food. Cages were maintained at 25 °C and 60% humidity, in 12 hr:12 hr light:dark cycles. Every two to three days, fresh food was provided and the number of dead flies was recorded. Each demography was replicated in at least two independent blocks. Statistical analysis was performed using the Kaplan-Meier survival log-rank test in IBM SPSS Statistics 21 as specified in the figure legends. For hyperoxia demographies female flies were placed in 25 × 95 mm vials containing 5 mL of either rapamycin treatment or vehicle control food. The vials were then placed into the hyperoxia chamber, with a constant perfusion of compressed O2 maintaining ambient air between 98 and 100% O2 at all times.
as measured by a ProOx P110 Oxygen Controller (BioSpherix, Lacona, NY). Vials were maintained at 25 °C in 12 hr:12 hr light:dark cycles. Every 4–6 hours, the number of dead flies was recorded. Each demography was replicated in at least two independent blocks. Statistical analysis was performed using the Kaplan-Meier survival log-rank test in IBM SPSS Statistics 21 as specified in the figure legends.

4.7. Larvae survival

30 adult males and adult females of two strains of \textit{sdhA}\textsuperscript{W;FRT42DPCW747AsdhA\textsuperscript{1404/CyOKrGFP}} and \textit{w;FRT42DSdhA\textsuperscript{1/CyOKrGFP}} were placed into grape juice-agar plates chambers for 12 hours in order to lay eggs. Immediately following this period, 55 to 60 eggs were transferred from the agar plate systems into plates of rapamycin at concentrations specified in the figures. The development and survival of mutants were determined using the Olympus GFP microscope, with the number of \textit{Drosophila} alive each day. Homozygous \textit{sdhA} mutant larvae were distinguished through the lack of expression of green fluorescent protein (GFP). Statistical analysis was performed using the Kaplan-Meier survival log-rank test in IBM SPSS Statistics 21 as specified in the figure legends.

4.8. Mitochondrial oxygen consumption

A mitochondrial suspension was prepared in the same manner as described above for the SDH enzymatic assay. This suspension was then assayed for the rate of oxygen consumption using an Oxytherm liquid-phase respirometer (Hansatech, Norfolk, UK) as described in [29]. In brief, respiration was measured by adding 5 mM of pyruvate and 5 mM malate as substrates to an isolated mitochondrial suspension in 1 ml of respiration buffer held in the respiration chamber at 30 °C. 125 nmol of ADP was added to generate state 3 respiration rates. All data was collected using Oxygraph Plus 2.1 software from Hansatech and imported into Microsoft Excel 2010 for analysis. Rates were standardized to protein content using a Bradford assay (Sigma-Aldrich, St. Louis, MO) following the manufacturer’s protocol. Data is presented as oxygen consumption under rapamycin treatment divided by the oxygen consumption of vehicle control treated mitochondria. Significance was determined by a Wilcoxon signed rank test in IBM SPSS Statistics 21.

Declarations

Author contribution statement

Frances Fan, Rheba Sam, Katherine Alvarado: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data.

Emma Ryan: Performed the experiments; Analyzed and interpreted the data.
Eugenia Villa-Cuesta: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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