RESEARCH ARTICLE

TORC1 Inhibition by Rapamycin Promotes Antioxidant Defences in a Drosophila Model of Friedreich’s Ataxia

Pablo Calap-Quintana¹, Sirena Soriano¹,2, José Vicente Llorens¹*, Ismael Al-Ramahi², Juan Botas², María Dolores Moltó¹,3*, María José Martínez-Sebastián¹

¹ Department of Genetics, University of Valencia, Burjassot, Valencia, Spain, 2 Department of Molecular and Human Genetics, Baylor College of Medicine, Houston, Texas, United States of America, 3 CIBERSAM, INCLIVA, Valencia, Spain

¤ Current address: Evolutionary Biology Center, Uppsala University, Uppsala, Sweden
* dmolto@uv.es

Abstract

Friedreich’s ataxia (FRDA), the most common inherited ataxia in the Caucasian population, is a multisystemic disease caused by a significant decrease in the frataxin level. To identify genes capable of modifying the severity of the symptoms of frataxin depletion, we performed a candidate genetic screen in a Drosophila RNAi-based model of FRDA. We found that genetic reduction in TOR Complex 1 (TORC1) signalling improves the impaired motor performance phenotype of FRDA model flies. Pharmacologic inhibition of TORC1 signalling by rapamycin also restored this phenotype and increased the lifespan and ATP levels. Furthermore, rapamycin reduced the altered levels of malondialdehyde + 4-hydroxyalkenals and total glutathione of the model flies. The rapamycin-mediated protection against oxidative stress is due in part to an increase in the transcription of antioxidant genes mediated by cap-n-collar (Drosophila ortholog of Nrf2). Our results suggest that autophagy is indeed necessary for the protective effect of rapamycin in hyperoxia. Rapamycin increased the survival and aconitase activity of model flies subjected to high oxidative insult, and this improvement was abolished by the autophagy inhibitor 3-methyladenine. These results point to the TORC1 pathway as a new potential therapeutic target for FRDA and as a guide to finding new promising molecules for disease treatment.

Introduction

Friedreich’s ataxia (FRDA), an autosomal recessive disease, is the most common inherited ataxia among Caucasians [1]. It is a multisystemic disease affecting the central and peripheral nervous systems and other non-neural organs, resulting in multiple signs and symptoms [2]. This incapacitating condition exhibits an irreversible progression that confines a patient to a wheelchair and leads to early death. FRDA is caused by a partial loss of FXN function [3], with the vast majority of patients carrying an intronic GAA expansion mutation in both alleles of
FXN codifies frataxin, a mitochondrial protein that is highly conserved through evolution [5] and whose deficiency results in several biochemical disturbances. Major alterations include impaired iron-sulphur cluster biogenesis, dysfunction of respiratory chain complexes and aconitase, mitochondrial iron accumulation and increased oxidative stress sensitivity [6].

A growing amount of data from patient samples and different model organisms of the disease suggest that oxidative stress plays an important role in the pathophysiology of FRDA. Biomarkers of oxidative damage, such as lipid peroxidation products, have been frequently found in patient samples and in a Drosophila model of the disease [7,8]. Increased levels of reactive oxygen species (ROS) have also been reported in FRDA lymphoblasts [9] and in mouse, Drosophila and yeast models [10–12]. In addition, frataxin depletion enhances the sensitivity to different pro-oxidant agents in FRDA cells, mice, Drosophila melanogaster, Caenorhabditis elegans and yeast [13–17]. Furthermore, a reduction in the ability to promote antioxidant defences has been reported in cultured fibroblasts from FRDA patients [18] and in the dorsal root ganglia from YG8R frataxin-deficient mice [19].

To date, there is no cure for FRDA, but several strategies for the discovery of effective therapeutics are being developed or tested in clinical trials (http://www.curefa.org/pipeline.html). These strategies seek to increase frataxin expression and to reduce the biochemical consequences of its deficiency, such as oxidative damage. Important progress has been achieved in frataxin replacement therapies [20,21], as well as in treatments directed to increasing protein levels [22]. Genetically manipulable organisms such as Drosophila are acquiring increased significance for medical and pharmaceutical research as valuable tools for testing potential therapies. The identification of the Drosophila FXN ortholog, fh, [23] led to the development of fly models of FRDA that can be used to explore frataxin function [24] and to provide in vivo evidence of a functional equivalence for human and fly frataxins [25]. These models have contributed to a comprehensive characterisation of the phenotype associated with frataxin deficiency [8,11,15,26–28]. Recently, we have validated the use of Drosophila as an experimental tool to screen for therapeutic molecules to treat FRDA and proposed that early treatments using the antioxidant idebenone and the iron chelator deferiprone may be advantageous to slow down the disease progression [29]. In addition the molecule methylene blue has been suggested for the treatment of the heart dysfunction in FRDA [30]. These findings stimulate further work using Drosophila to find new pharmacological drugs that may be relevant to this disease.

Here, we conducted a genetic screen of candidate genes related to FRDA pathophysiology to identify new therapeutic targets for this disease. We found that downregulation of TOR Complex 1 (TORC1) function suppresses the impaired motor performance of our Drosophila model of FRDA [15]. To evaluate the therapeutic efficacy of TORC1 inhibition, we used rapamycin, a lipophilic macrolide that acts as an inhibitor of the TOR kinase [31]. This treatment was able to increase the motor performance and survival of frataxin knockdown flies and could also induce an improvement in the oxidative status and an increase in the ATP levels.

**Materials and Methods**

**Drosophila melanogaster strains**

The UAS-fhRNAi line was previously generated in our laboratory and produces a reduction of up to 70% of frataxin mRNA when expressed ubiquitously using the actin-Gal4 driver; this reduction is compatible with a normal development [15]. The y w, w<sup>1118</sup> UAS-GFP, actin-Gal4, nos-Gal4; UAS-GFP-LC3, UAS-fxo-GFP, cnc-EGFP, UAS-S6k<sup>STDETE</sup> (here referred as S6k<sup>CA</sup>) and Thor<sup>2</sup> (here referred as 4E-BP<sup>LOF</sup>) strains were obtained from the Bloomington
Stock Center. $y^w$, $actin-Gal4$ and $w^{118}$; $actin-Gal4$ flies were used as controls, while UAS-fhRNAi; $actin-Gal4$ flies were used as FRDA model flies (here referred as fhRNAi).

Culture conditions and drug treatments

*Drosophila* stocks were maintained at 25°C under a 16/8 hour light/dark cycle on standard cornmeal agar medium. The media named “RAP”, “3-MA” and “RAP + 3-MA” were prepared with, respectively rapamycin at 1 μM (LC Laboratories), 3-methyladenine at 67 μM (Sigma-Aldrich), and both rapamycin at 1 μM and 3-MA at 67 μM. All compounds were previously dissolved in dimethylsulfoxide (DMSO; Sigma-Aldrich) at a final concentration of 0.1% (v/v). The medium named “DMSO” only contained this compound at 0.1% (v/v) and was used as control medium. Crosses were conducted at 25°C in the supplemented media. F1 flies of the appropriate genotype were transferred to fresh vials containing the compound every 3 days.

Genetic screen

The UAS-fhRNAi; $actin-Gal4$ flies (fhRNAi flies) were crossed at 28°C with approximately 300 lines, including RNAi lines from the Vienna Drosophila Resource Center and loss-of-function and overexpression lines for candidate genes from the Bloomington Stock Center. We focused on candidate pathways implicated in FRDA pathophysiology comprising metal homeostasis, response to oxidative stress, apoptosis and autophagy. Motor performance tests were conducted as described previously [32] for the identification of genetic modifiers of frataxin depletion. We recorded the number of flies that climbed to a height of 11.5 cm.

Climbing and survival assays

Groups of fifteen 7-day-old males were transferred into vials of 1.5 cm in diameter and 25 cm in height. The height reached from the bottom of the vial by each fly in a period of 10 s was recorded with a camera. For each genotype, approximately 100 flies were tested. The results are expressed in percentage, taking as 100% the mean climbing speed of control flies in the DMSO medium. Lifespan was measured starting with 100 adult males of each genotype and by recording the number of living flies every 3 days. Survival under hyperoxia was measured using 30 adult males exposed to a constant flux of 99.5% oxygen under a low positive pressure from day 1 to day 4 after eclosion from the puparium. Three replicates were performed, and the results showed the percentage of dead flies after 4 days of hyperoxia.

GFP-LC3 quantification in larvae

The UAS-fhRNAi and $y^w$ lines were crossed with the nos-Gal4; UAS-GFP-LC3 strain, which expresses the microtubule-associated protein 1A/1B-light chain 3 (LC3) as a fusion protein with GFP under the control of the nanos promoter (a marker for autophagy [33]). Fat bodies of third instar larvae were imaged with a fluorescence microscope (Leica DM 2500; Leica Microsystems) using a x40 objective. Larvae were maintained in normoxia or subjected to one day of hyperoxia before the dissections. The number of fluorescent dots per field was counted automatically using the tools of ImageJ software (National Institutes of Health, USA). The results are expressed in percentage, taking as 100% the average number of dots for control flies in the DMSO medium.

Biochemical assays

Biochemical assays were conducted in triplicate or quadruplicate with thirty 7-day-old males of the appropriate genotype. ATP levels were determined using the ATP Detection Reagent of
the Mitochondrial ToxGlo Assay (Promega). Flies were homogenised in a buffer of 0.25 M Sucrose; 10 mM HEPES-NaOH pH 7.4; 0.1% Triton X-100 (v/v), Na3VO4 5 mM, and the extract was centrifuged at 1000 g for 10 min at 4°C. The luminescence of the supernatant was measured using a Tecan Infinite M200 PRO luminometer (Tecan Group). ATP levels were normalised to the total protein, which was measured using the BCA assay. The results are expressed in percentage, taking as 100% the ATP level of control flies in the DMSO medium.

The concentration of malondialdehyde (MDA) + 4-hydroxyalkenals (HAE) was measured using the Bioxytech LPO-586 Kit (Oxis International). Flies were homogenised in a buffer of 50 mM Tris-HCl at pH 7.4 with 5 mM butylated hydroxytoluene, and the extract was centrifuged at 3000 g for 10 min at 4°C. A586 measurements were performed in a Spectronic Genesys 5 spectrophotometer (Milton Roy). MDA + HAE levels were normalised to the protein amount determined by the Bradford assay. The results are expressed in nmol of MDA + HAE per μg of protein.

The total concentration of GSH, including both the reduced and oxidised forms, was measured using a Bioxytech GSH-420 Spectrophotometric Assay Kit (Oxis International). Flies were homogenised in trichloroacetic acid, and the extract was centrifuged at 3000 g for 10 min at 4°C. A420 was measured in a Spectronic Genesys 5 spectrophotometer (Milton Roy). The obtained data were normalised to the total protein determined by the Bradford assay, and the results are expressed in mmol of total GSH per μg of protein.

For measurements of the aconitase activity in hyperoxia, flies were incubated in normoxia for 5 days and then treated with 99.5% oxygen for 2 days. Aconitase activity was determined from the whole-fly extracts using the Bioxytech Aconitase-340 Spectrophotometric Assay Kit (Oxis International) as previously described [29]. A340 was measured using a Tecan Infinite M200 PRO luminometer (Tecan Group), and the results are expressed in percentage, taking 100% the aconitase activity of control flies in DMSO medium.

Quantitative real-time PCR (RT-qPCR)

Total RNA was extracted from 7-day-old males using a miRNeasy Mini Kit (Qiagen). RNA was converted into cDNA with Expand Reverse Transcriptase (Roche) and oligo-dT primers. Amplification was conducted using the Step One Plus Real-Time PCR System (Applied Biosystems) and Power SYBR Green (Applied Biosystems). The following primers were used for the transcript amplification of the different genes:

- frataxin homolog (fh), 5'ACACCCCTGAGCAGCCGACGACG3' and 5'CCAGGTTTCAAGGTGTTAGCACGAC3';
- Adenylyl cyclase 76E (Ac76E), 5'CGATCAAATAGCTCAGGAGAACCA3' and 5'CATTATGCCGTCGTCATCA3';
- cap-n-collar (cnc), 5'CACGGTTTTCCAAGTCACCACAC3' and 5'TCCCTGCAAGCACACACAT3';
- Catalase (Cat), 5'GGTGGATGTGTTCTAAATCTCTGTGTT3' and 5'GGTGTAATGCGACCACAGAGAA3';
- forhead box sub-group O (foxo), 5'CCACCGCCAATACAAAC3' and 5'CCTGGCCAGCCCAAAAGATA3';
- Glutamate-cysteine ligase catalytic subunit (Gclc), 5'GACGGCGGAAACAGAGTGCACG3' and 5'GAATCGATGTGAGCCATGCT3';
- Glutathione S transferase D1 (GstD1), 5'TACATCGCGAGTTACAACAG3' and 5'CAGGTTGACGAGCCTTCTGTT3';
- Peroxiredoxin 3 (Prx3), 5'CCGATTTCAAGGTGCCACTGCT3' and 5'ACACCAATTTCCGGTGGGCA3';
- Sestrin (Sesn), 5'CCCCAGTCCACGATCATCTT3' and 5'CCGGTCCACCAGACGAC3';
- Superoxide dismutase (Sod), 5'AACTGAAGTACGGCCATGCT3';
- Superoxide dismutase 2 (Sod2), 5'CAAGATAGTACGGACGCTCAG3' and 5'CGCGCTGATTAGGCTCCAGG3'.

The Ribosomal protein L32 gene (RpL32) was used as an internal control and was amplified using the 5'CCAAGCATTGTATCCTCCGAAC3' and 5'GCCGGTGCCTGTTGATCCGCT3' primers. The results were analysed using the Step One Plus software v2.0 (Applied Biosystems). The gene
expression levels are relative to the internal control, and the relative quantification of each cDNA was calculated in quadruplicate experiments using the Ct method. The results are expressed as the fold change of relative gene expression compared with that for control flies in the DMSO medium.

Nuclear isolation and GFP quantification

fhRNAi flies were crossed with the UAS-foxo-GFP and cnc-EGFP lines. For UAS-foxo-GFP, third instar larvae were collected as the co-expression with fhRNAi resulted in adult semi-lethality; whereas 7-day-old males were analysed for cnc-EGFP. Flies with the genotypes actin-Gal4; UAS-foxo-GFP and actin-Gal4; cnc-EGFP, respectively, were used as controls. Total extractions and nuclear fractions were isolated following the procedure described previously [34], and GFP fluorescence was measured using a Tecan Infinite M200 PRO luminometer (Tecan Group). The results are expressed as the nuclear/total fluorescence ratio, considering the ratio of the control flies in the DMSO medium as 1.

Statistical Analysis

Statistical analyses were performed using GraphPad Prism 5.03 software (GraphPad software). Kaplan-Meier survival plots were analysed using semiparametric log rank tests. For the comparison of means, we performed an unpaired nonparametric Student’s t test. In all cases, values of P<0.05 were considered statistically significant. Error bars represent standard error of the mean (SEM).

Results

TORC1 pathway genetically interacts with frataxin

To identify genetic modifiers that might modulate the phenotypes caused by frataxin knock-down in Drosophila, we conducted a genetic screen of candidate pathways implicated in FRDA pathophysiology. Specifically, we examined the effect of knockdown, loss and gain-of-function alleles corresponding to metal homeostasis, response to oxidative stress, apoptosis and autophagy pathways. We then conducted motor performance tests to determine whether these alleles suppress the motor impairment of the fhRNAi flies. The screen revealed four modifiers from the TORC1 signalling pathway: the tuberous sclerosis complex protein 1 (Tsc1), the protein kinase S6K (S6k), the eukaryotic translation initiation factor 4E (eIF-4E) and the Leucine-rich repeat kinase (Lrrk) (Fig 1A).

One of the most important regulators of TORC1 activity is the tuberous sclerosis complex (TSC), which is a heterodimer that comprises the proteins TSC1 and TSC2. TSC1/2 negatively regulates TORC1 signalling by inactivating the Ras homolog enriched in brain ortholog (Rheb) GTPase. The simultaneous knockdown of Tsc1 and frataxin resulted in semi-lethality, whereas the expression of the RNAi for Tsc1 with the actin-Gal4 driver had no effect on viability in control flies. S6k and eIF-4E are downstream targets of TORC1. Expression of a dominant-negative form of S6K [35] improved the motor performance of the fhRNAi flies (Fig 1B). In contrast, the expression of a constitutively active version of S6K [35] produced a detrimental effect when combined with frataxin knockdown by inducing semi-lethality. Regarding eIF-4E, a loss of function mutation suppressed the impaired motor performance phenotype of the fhRNAi flies (Fig 1C). In all cases, the S6k and eIF-4E alleles on their own had no effect on the viability or motor performance of control flies. We also demonstrated that knocking down the Lrrk suppresses the frataxin knockdown phenotype (Fig 1D). Lrrk is the Drosophila ortholog of the human LRRK2, and dominant pathogenic mutations in this gene cause the most common
familial forms and some sporadic cases of Parkinson’s disease [36]. In vitro studies show that the eIF-4E binding protein (4E-BP) is a substrate of Lrrk [37], (Fig 1A). Genotypes of the Drosophila strains corresponding to these genetic interactors are shown in S1 Table.

Rapamycin improves the motor performance and lifespan deficits of the frhRNAi flies

Because the reduction of TORC1 activity decreases S6K and eIF-4E activities [38], we tested whether pharmacologic inhibition of this complex with rapamycin would also improve the...
phenotypes of our *Drosophila* FRDA model. First, we tested the effect of 1 μM rapamycin on the motor performance of the frataxin knockdown flies. This concentration had been previously proved effective in *Drosophila* [39] and did not provoke the negative effect on viability that we found for the higher concentrations. Here, we used the climbing speed of flies as it provides a more accurate assessment of the motor performance. In DMSO medium, 7-day-old *fh* RNAi flies showed a 25% decrease in climbing speed compared with controls raised in the same medium. Rapamycin induced the recovery of the motor performance phenotype of the frataxin knockdown flies up to control levels (Fig 2A). Because another feature of our *Drosophila* model of FRDA is a shortened lifespan [15], we also tested the effect of rapamycin on this phenotype. It has been reported that rapamycin increases the lifespan in *Drosophila* and other organisms [40]. Accordingly, 1 μM rapamycin produced a slight but statistically significant increase in the lifespan of both control (*P* = 0.0116) and *fh* RNAi (*P* = 0.0004) flies (Fig 2B).

To confirm the inhibitory effect of rapamycin on TORC1 activity, we measured the developmental time needed by flies to reach the adult stage. It has been shown that, in conjunction with the insulin/IGF signalling pathway, TORC1 controls the larval development in *Drosophila*, matching the speed of growth to the nutrient availability. A reduction in the amount of food reduces TORC1 signalling in the fat body and the prothoracic gland and increases the time needed by the individuals to reach the pupae stage [41]. We observed that the rapamycin treatment increased, by approximately one day, the mean time needed by both control and *fh* RNAi individuals to reach the adult stage (S1 Fig). This result indicated that rapamycin reduces TORC1 signalling similarly to the food restriction effect and that the rapamycin concentration used could efficiently modify the TORC1 activity.

Finally, to test whether the suppression by rapamycin of the motor performance and lifespan phenotypes of *fh* RNAi flies was an artefact caused by interference with the GAL4/UAS system, we verified that rapamycin had no effect on the expression of a GFP reporter (S2 Fig). RT-qPCR of the transcript for frataxin showed that rapamycin did not alter the level of the *fh* mRNA either in the control or in the frataxin knockdown flies (S3 Fig).

Rapamycin protects against oxidative stress in the FRDA model

Oxidative stress plays a central role in the pathophysiology of FRDA, as shown in patients and in cellular and animal models of the disease [7–12], including our *Drosophila* model. In this context, the impairment of motor performance and survival exhibited by the *fh* RNAi flies were ameliorated after treatment using the antioxidant idebenone [29]. *fh* RNAi flies also show an increased sensitivity to external oxidative stress, as indicated by an enhanced reduction in motor performance and lifespan [15]. Taking into account these data, we asked whether rapamycin might be suppressing the FRDA toxicity in part by decreasing oxidative stress.

We monitored the effect of rapamycin on the levels of malondialdehyde (MDA) + 4-hydroxyalkenals (HAE) and total glutathione, two markers of oxidative stress. As expected, *fh* RNAi flies show a higher amount of MDA + HAE compared with that of control flies in the DMSO medium (Fig 3A). Interestingly, rapamycin restored the MDA + HAE levels in the *fh* RNAi flies, whereas rapamycin had no effect on the controls. As shown in Fig 3B, *fh* RNAi flies had higher levels of total glutathione than did control flies and rapamycin produced a significant reduction in the total amount of glutathione in the *fh* RNAi flies but did not affect the total glutathione levels in the controls. Therefore, the inhibition of TORC1 with rapamycin seems to
Fig 2. Rapamycin increases climbing speed and survival. (A) Rapamycin treatment increases the climbing speed of 7-day-old males in both the control and FRDA fly groups, and this effect is not affected by the addition of the autophagy inhibitor 3-MA. A constitutively active allele of S6K (S6kCA) and a loss of function allele of 4E-BP (4E-BPLOF) prevent rapamycin from increasing the climbing speed. Climbing speed is expressed in percentage (mean climbing speed of control flies in the DMSO medium is 100%). ns: non-significant, \(*P<0.05\), \(**P<0.01\), \(***P<0.001\). Error bars represent SEM. (B) Survival is also increased in control \((P = 0.0116)\) and FRDA flies \((P = 0.0004)\) after the drug treatment. Control \((y^{1} w^{+}; actin-Gal4 \) flies), control/S6kCA \((control \) flies carrying the S6kCA \) allele), control/4E-BPLOF \((control \) flies carrying the 4E-BPLOF \) allele), \(fhRNAi\) \((UAS-fhRNAi; actin-Gal4 \) flies), \(fhRNAi/S6kCA\) \((model \) flies carrying the S6kCA \) allele), \(fhRNAi/4E-BPLOF\) \((model \) flies carrying the 4E-BPLOF \) allele).

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ameliorate the oxidative stress injury in frataxin knockdown flies, resulting in a decrease in the altered MDA + HAE and total glutathione levels.

**Autophagy induction by rapamycin is not critical except in hyperoxic conditions**

It is well established that rapamycin treatment leads to the activation of autophagy through the inhibition of TORC1 [31]. Thus, we asked whether autophagy might play a role in the protection against oxidative stress conferred to the *fh* RNAi flies by rapamycin. For this purpose, we used the 3-MA compound, which inhibits Vps34 (vacuolar protein sorting 34), a class III phosphoinositide 3-kinase that is essential for autophagosome biogenesis [42]. First, we confirmed that at the concentrations used in this study, rapamycin and 3-MA were effective as inducers and inhibitors of autophagy, respectively. As shown in S4 Fig, rapamycin induces the formation of autophagosomes, which were labelled with GFP-LC3, in control and frataxin knockdown flies, and the addition of 3-MA decreased the number of GFP-LC3 dots.

Next, we tested the effect of autophagy inhibition on the levels of MDA + HAE and total glutathione. No changes were detected between the RAP and the RAP + 3-MA media (Fig 3A).

![Graphs showing the effect of autophagy induction by rapamycin on oxidative stress protection](image)

**Fig 3. Rapamycin increases oxidative stress protection.** In the normoxic condition, rapamycin reduces the altered levels of malondialdehyde + 4-hydroxyalkenals (A) and total glutathione (B) of model flies without requiring autophagy induction as the addition of 3-MA has no effect. In hyperoxia, rapamycin improves the survival of FRDA flies (C) and increases the aconitase activity (D). The addition of 3-MA abolishes these effects in model flies, highlighting the relevance of autophagy in this highly oxidative condition. Control (*y* w*; actin-Gal4 flies), *fh* RNAi (UAS-*fh* RNAi; actin-Gal4 flies). ns: non-significant, *P*<0.05, **P*<0.01, ***P*<0.001. Error bars represent SEM.

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and 3B), indicating an autophagy-independent effect for rapamycin. In addition, the beneficial effect of rapamycin on the motor performance was also autophagy-independent (Fig 2A). These data show that even though autophagy is induced by rapamycin, autophagy has no important protective effect in these conditions.

Then, we asked whether autophagy induction by rapamycin is beneficial for flies under external oxidative stress. We assessed the effect of rapamycin on the survival of fhRNAi flies incubated in a hyperoxic atmosphere for 4 days. In the DMSO medium, we observed higher mortality in fhRNAi flies (28%) than in controls (6%). Rapamycin reduced the number of dead fhRNAi flies but had no significant effect on the survival of the controls (Fig 3C). Interestingly, the decreased lethality observed in hyperoxia conditions was abolished by the addition of 3-MA (Fig 3C), suggesting that autophagy is indeed necessary for the protective effect of rapamycin on fhRNAi flies in hyperoxia. In this condition, rapamycin and 3-MA were also effective as an inducer and inhibitor of autophagy, respectively (S4 Fig).

To further confirm this hypothesis, we measured the activity of aconitase during hyperoxia. Under this experimental condition, the reduction of enzyme activity was significantly higher in the fhRNAi flies than in the control flies [15]. We observed that the aconitase activity increased in the rapamycin-treated fhRNAi flies and that this increase was also abolished by the addition of 3-MA (Fig 3D). Together, these data suggest that the autophagy induction by rapamycin is required for the protection against highly oxidative conditions; however, other mechanisms downstream of TORC1 should act in conditions of endogenous production of ROS in the FRDA model.

**Rapamycin enhances antioxidant defences increasing the nuclear translocation of Cnc**

TORC1 modulates the function of several transcription factors that, in turn, control the transcription of important antioxidant genes [43,44]. To determine whether rapamycin transcriptionally induces endogenous antioxidant defences, we analysed the expression of two key transcription factors implicated in antioxidant protection (foxo and cnc) and four well-known target genes (Sesn and Ac76E for FOXO; Gclc and GstD1 for Cnc). In the DMSO medium, we observed higher levels of the foxo (53% increase) and cnc (38% increase) transcripts in the fhRNAi flies than in the controls. Rapamycin did not modify the expression of these genes at the transcriptional level (Fig 4A and 4D). The FOXO target genes Sesn and Ac76E showed no differences between the control and fhRNAi flies in both DMSO and RAP medium (Fig 4E and 4F). In contrast, the transcript levels of the Cnc target genes were higher in the fhRNAi flies than in the controls when both were cultured in the DMSO medium (40% for Gclc and 34% for GstD1). Rapamycin increased the expression of these genes in both the control and fhRNAi flies (Fig 4B and 4C). Rapamycin also increased the mRNA level of Cat, Prx3, Sod and Sod2 (Fig 4G–4J), which encode important enzymes that protect the cell from oxidative damage and are subjected to overlapping regulation of FOXO and Cnc transcription factors.

To explain the augmented expression of the Cnc target genes by rapamycin, we searched for cellular localisation of Cnc using an EGFP-tagged cnc allele. We found a higher nuclear/cellular fluorescence ratio of Cnc-EGFP after rapamycin treatment in both the control and fhRNAi flies (Fig 4K). No differences were observed in the case of a FOXO-GFP fused protein (S5 Fig). These results indicate that rapamycin enhances the protection against oxidative stress by inducting endogenous antioxidant defences and that this effect is mediated, at least in part, by an increase in the nuclear translocation of the transcription factor Cnc.
Rapamycin increases the availability of ATP through 4E-BP

A pathological reduction of frataxin levels results in an impairment of ATP synthesis [45,46]. We measured the ATP levels in whole-fly extracts, and we did not find significant differences when comparing \( fh \) RNAi and control flies in the DMSO medium (Fig 5). Because different Drosophila tissues have distinct sensitivity to frataxin depletion [15], it is possible that the reduced ATP levels may be restricted to these tissues. Interestingly, rapamycin treatment increased the ATP levels (Fig 5) in both the control (41% increase) and frataxin knockdown flies (37% increase). This increase may contribute to the beneficial effect of rapamycin on the FRDA phenotype. To identify the pathway downstream of TORC1 involved in the ATP increase, we combined the rapamycin treatment with 3-MA, a constitutively active allele of S6K or a loss of function allele of 4E-BP, and we measured the ATP levels in the three cases. We observed that the 4E-BP mutation prevented rapamycin from increasing the ATP levels.
while the expression of the constitutively active S6K and the inhibition of autophagy had no effect on that increase. These results indicate that rapamycin ameliorates the ATP availability in flies through the 4E-BP and that inactivation of S6K or autophagy induction after TORC1 inhibition is not critical in this process [47,48]. Nevertheless, the modulation of the activity of both S6K and 4E-BP by TORC1 but not autophagy is indeed required for the rapamycin-mediated recovery of motor performance of \( \text{fh} \) RNAi flies (Fig 2A).

Discussion

TOR is an evolutionarily conserved protein that senses and integrates various environmental and intracellular signals to regulate growth and homeostasis in all eukaryotic cells. It functions by forming two structurally and functionally different multiprotein complexes, TORC1 and TOR Complex 2 (TORC2). TORC1 is better described and regulates many major cellular functions, including protein synthesis, lipid biogenesis and autophagy. TORC1 is sensitive to inhibition by rapamycin treatment [49].

In this study, we identified several components of the TORC1 pathway as modifiers of frataxin knockdown phenotypes in *Drosophila melanogaster*. We found that a genetic reduction in TORC1 signalling activity suppresses the impaired motor performance phenotype of the \( \text{fh} \) RNAi flies. Accordingly, genetic activation of TORC1 signalling produced a detrimental effect when combined with frataxin knockdown by inducing semi-lethality. Thus, we tested the therapeutic usefulness of a pharmacologic inhibition of TORC1 in the \( \text{fh} \) RNAi flies, using the
natural macroline rapamycin [31]. We observed that rapamycin restored the motor performance of frataxin knockdown flies to normal levels, in agreement with the genetic reduction of S6k and elf-4E. Rapamycin also increased the mean and maximum survival of the fhRNAi flies similarly as it did in controls. It is well known that TORC1 inhibition prolongs the lifespan of different species [40,50] and such effect is extended to fhRNAi flies.

The expression of dominant-negative forms of TOR and S6K and TOR inhibition by rapamycin provides flies with resistance to oxidative stress, whereas increased Rheb-TOR-S6K signalling sensitises flies to this type of stress and promotes early senescence of locomotor activity [50, 51]. Furthermore, it has also been reported in Drosophila that 4E-BP is important for survival under different types of stress, including oxidative insult [52]. In particular, high levels of oxidative stress markers have been found in FRDA patient samples as well as in several models of the disease [9,53–55]. In agreement with these results, we found an increased amount of MDA and HAE in the fhRNAi flies; these lipid peroxidation compounds are produced after the breakdown of unstable polyunsaturated fatty acid peroxides. The fhRNAi flies also showed increased levels of total GSH, a molecule with antioxidant function that reduces hydrogen and lipid peroxides when it is oxidised to its disulphide form, GSSG. Interestingly, rapamycin reduced significantly the MDA + HAE and total glutathione levels in the fhRNAi flies, restoring to some extent the normal situation observed in control flies. Recently, a similar result has been reported in the yeast frataxin knockout model in which rapamycin reduces ROS production [56].

The inhibition of TOR signalling by rapamycin has been shown to be protective against toxicity in several cell and animal models of neurodegenerative diseases. In some cases, this protective effect of rapamycin appears to be autophagy-dependent, particularly in neurodegenerative diseases associated with aggregation-prone mutant proteins [57]. However, our results indicated that in a normoxic condition, autophagy is not the main mechanism by which rapamycin protects fhRNAi flies against the ROS injury caused by frataxin deficiency. Our data show that neither the rescue of motor performance nor the protection against ROS induced by rapamycin were affected by the chemical inhibitor of autophagy 3-MA in normoxia.

TORC1 inhibition by rapamycin increases the transcript levels of genes involved in the free radical scavenging and Nrf2-mediated oxidative stress response in mouse adult stem cells [43] and in C. elegans [44]. We studied the possible effect of rapamycin on the activity of the transcription factors FOXO and Cnc (the Drosophila ortholog of the mammalian Nrf2), which control the expression of many genes involved in resistance against different types of stresses [44,58]. Our results showed that rapamycin increased the transcription of antioxidant genes dependent upon Cnc but not FOXO, and that this effect is mediated by an increase in the nuclear translocation of Cnc. Therefore, it may be the origin of the protective effect of rapamycin against oxidative stress caused by frataxin reduction in the fhRNAi flies. There are still many aspects of Nrf2 regulation that remain poorly understood. Nevertheless, several regulation mechanisms have been already described, both dependent and independent of the Keap1 protein (recently reviewed in [59]). Some of these mechanisms could explain the increase in Cnc activity mediated by rapamycin. Protein kinase C is able to disrupt the association between Nrf2 and Keap1, promoting the translocation of Nrf2 into the nucleus. GSK3β promotes Nrf2 ubiquitination and the degradation of the protein by the proteasome; thus, GSK3β inhibition can also contribute to Nrf2 activity. Both Protein kinase C activation and GSK3β inhibition can be triggered by an increase in PI3K-Akt signalling, and rapamycin is able to produce this effect by means of a negative feedback loop in TORC1 regulation [60,61] (S6 Fig). However, the actual mechanism by which rapamycin increases Cnc activity in FRDA model flies needs further research.
Interestingly, we found that autophagy becomes an important protective mechanism in \( f_h \)RNAi flies subjected to a strong external oxidative insult. \( f_h \)RNAi flies cultured in a hyperoxic environment show an enhanced reduction of motor performance and lifespan [15]. In this work, we observed that rapamycin improves the survival and aconitase activity of \( f_h \)RNAi flies subjected to hyperoxia and that the beneficial effect of rapamycin decreases when 3-MA is added. Our results agree with other studies [39,50,62] in which autophagy is induced by rapamycin or by overexpressing/interfering \( Atg \) genes or components of the TORC1 signalling cascade to protect flies against strong external oxidative stress. Altogether, these data suggest that the autophagy induction by rapamycin operates as a cellular mechanism to protect against strong oxidative insults. However, in the oxidative conditions resulting from the endogenous ROS production in the \( f_h \)RNAi flies, the protective effects of rapamycin are more likely to reside in its antioxidant properties rather than autophagy induction.

In conditions of frataxin depletion, several deficiencies in the mitochondrial electron transport chain result in impaired generation of ATP [45,46]. Reducing TORC1 activity may be beneficial for the energy status of frataxin-depleted cells because this signalling pathway activates specific regulatory mechanisms that can increase mitochondrial efficiency. Bonawitz et al. [47] reported that \( tor1 \) null yeast exhibit a higher rate of mitochondrial translation and steady-state abundance of several mitochondria-encoded OXPHOS components. In \textit{Drosophila}, dietary restriction, whose effects are mediated to a great extent by TORC1, is capable of increasing the translation of genes involved in oxidative phosphorylation to ensure continued ATP generation, and this effect has been attributed to the TORC1 target 4E-BP [48]. In support of this idea, we found that inhibition of TORC1 by rapamycin increases the total ATP levels of both control and \( f_h \)RNAi flies, which may contribute to the recovery of the motor performance and the slight increase in lifespan of the \( f_h \)RNAi flies. We also found that 4E-BP is the key mediator in the increase of ATP levels after TORC1 inhibition by rapamycin. Finally, although much progress has been made in the understanding of TORC1 function, we cannot exclude the possibility that other unknown molecular mechanisms regulated by this critical signalling complex may be contributing to the recovery of the motor dysfunction of the rapamycin-treated \( f_h \)RNAi flies.

Rapamycin is a well-described drug approved for human uses. This drug and its analogues (rapalogs) have important clinical applications in oncology and transplantation medicine. Ongoing clinical trials using rapalogs to treat different malignancies are providing an extensive body of data about the safety, tolerability and side effects of rapalogs [63]. In FRDA, lower doses of rapamycin may be beneficial combined with other drugs as antioxidants and iron chelators. It may enhance the advantages of either compound acting alone because none of the tested antioxidants or chelators has been proven to be sufficiently effective on the neurological symptoms of FRDA [64].

Our results show that the reduction of TORC1 signalling activity in the \textit{Drosophila} model of FRDA rescues several phenotypes (impairment of motor abilities and reduced lifespan) that mimic the clinical features of this disease. These results point to the TORC1 pathway as a new potential therapeutic target for FRDA and as a guide to finding new promising molecules for disease treatment.

**Supporting Information**

**S1 Fig.** TORC1 inhibition by rapamycin increases development time to adulthood in control and \( f_h \)RNAi flies. The time needed by individuals to eclose from the puparium was measured. The day the crosses were made was established as day zero. Parental flies were maintained in these vials for 2 days and then were removed. The results indicate the average
time, in days, needed by individuals of F1 to complete the preadult development. Control (y1w; actin-Gal4 flies), fhRNAi (UAS-fhRNAi; actin-Gal4 flies). ns: non-significant, ***P<0.001.

S2 Fig. Rapamycin does not interfere with the GAL4/UAS system. Fluorescence from thirty 7-day-old males expressing GFP in a ubiquitous pattern (UAS-GFP; actin-Gal4) was measured as previously described in [29].

(TIF)

S3 Fig. Rapamycin does not alter the level of fh mRNA neither in control nor in fhRNAi flies. Control (y1w; actin-Gal4 flies), fhRNAi(UAS-fhRNAi; actin-Gal4 flies). ns: non-significant.

(TIF)

S4 Fig. Effect of rapamycin and 3-MA on the induction and inhibition, respectively, of autophagosome formation. ns: non-significant, *P<0.05, **P<0.01, ***P<0.001. control/GFP-LC3 (UAS-GFP-LC3/+; Nos-Gal4/+) and fhRNAi/GFP-LC3: (UAS-GFP-LC3/UAS-fhRNAi; Nos-Gal4/+)

(TIF)

S5 Fig. Rapamycin does not alter the fraction of FOXO-GFP located in the nucleus. control/foxo-GFP (control flies expressing a foxo allele tagged with the GFP) and fhRNAi/foxo-EGFP: (fhRNAi flies expressing a foxo allele tagged with the GFP).

(TIF)

S6 Fig. Hypothesis of Nrf2 activation by rapamycin. Rapamycin might increase Nrf2 activity by mechanisms depending on PKC and GSK3β, triggered by a TORC1 negative feedback loop which may increase PI3K-Akt signalling.

(TIF)

S1 Table. Genotypes of the Drosophila strains corresponding to the genetic interactors in the TORC1 pathway.

(TIF)

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Author Contributions
Conceived and designed the experiments: PC-Q SS JVL IA-R JB MDM MJM-S. Performed the experiments: PC-Q SS JVL. Analyzed the data: PC-Q SS JVL IA-R JB MDM MJM-S. Contributed reagents/materials/analysis tools: IA-R JB MDM MJM-S. Wrote the paper: PC-Q SS MDM MJM-S. Revised the manuscript critically: JVL IA-R JB.

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