UNIVERSITÀ DEGLI STUDI DI PADOVA

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ELABORATO DI LAUREA

L'inserzione di ripetizioni GAA nel gene di fratassina di *Drosophila* con CRISPR/Cas9 fornisce un modello per lo studio dell'atassia di Friedreich e dell'effetto di protezione *in vivo* con N-acetil cisteina

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Indice

Abstract

L'atassia di Friedreich (FA) è causata da un'espansione della tripletta GAA nel primo introne del gene *FXN*, codificante la proteina fratassina, la cui espressione risulta diminuita. Il lavoro analizzato in questo elaborato di laurea ha lo scopo di studiare l'effetto dell'inserzione, mediante CRISPR/Cas9, di circa 200 GAA nell'introne del gene *fh* di *Drosophila melanogaster* (omologo di *FXN*). Al deficit d'espressione della proteina si associano ritardo nello sviluppo e elevata letalità. Per ovviare alla precoce letalità della mutazione, sono stati utilizzati *tools* genetici che hanno consentito di sovraesprimere la proteina durante lo sviluppo. L'RNA Seq ha evidenziato alterazioni nel metabolismo degli amminoacidi e marcatori di stress ossidativo. In particolare, si è notato un generale aumento dell'espressione di *Tspo*, proposto come biomarker per la malattia, totalmente riportata alla normalità dall'espressione di fratassina nell'adulto. Infine, è stato possibile valutare l'effetto positivo dell'N-acetil cisteina che aumenta il tasso di sopravvivenza e migliora le capacità locomotorie, la resistenza allo stress ossidativo e l'attività aconitasica. Questo studio evidenzia anche l'efficacia della tecnologia di *genome editing* CRISPR/Cas9 per introdurre mutazioni umane in geni ortologhi di *Drosophila*.

Stato dell'arte

Atassia di Friedreich: patologia e profilo genetico. L'atassia di Friedreich (FA), una forma di atassia recessiva, è una malattia neurodegenerativa progressiva causata dalla mutazione del gene *FXN*, codificante la proteina mitocondriale fratassina, una proteina ubiquitaria, la cui deficienza altera la biogenesi dei centri Fe-S, cofattori chiave della catena respiratoria, e la produzione di ATP mitocondriale, provoca un accumulo di ferro nel mitocondrio e aumenta la sensibilità delle cellule allo stress ossidativo (1). I sintomi insorgono nei pazienti a 10-15 anni e sfociano nella perdita delle capacità locomotorie e in problemi cardiaci, con riduzione dell'aspettativa di vita a 37 anni. La causa principale di morte nei pazienti affetti da FA è correlata a problemi cardiaci. Il 96 % dei pazienti affetti è omozigote per un'espansione ripetuta di triplette GAA nel primo introne del gene *FXN*, il restante 4 % è un eterozigote composto con l'espansione GAA in un allele e un'altra mutazione patogenica nell'altro. In un individuo sano in media sono presenti 36 ripetizioni GAA. Negli affetti invece si misurano da 56 a 1300 ripetizioni. Esiste una correlazione negativa tra dimensione dell'espansione e età di comparsa dei sintomi e di complicazioni gravi come la cardiomiopatia. L'espansione GAA nel gene *FXN* è instabile sia nella meiosi che nella mitosi. Il numero di ripetizioni può aumentare o diminuire nel passaggio alla progenie e studi sulla dimensione dell'espansione GAA nel sistema nervoso centrale, nel pancreas e nel tessuto cardiaco ne hanno rivelato l'instabilità mitotica. E' stato evidenziato inoltre l'incremento dell'instabilità post-zigotica con l'avanzamento d'età (2).

Silenziamento di *FXN*: ipotesi molecolare ed effetti. L'espansione GAA costituisce una porzione di cromatina a effetto repressivo che ostacola la trascrizione di *FXN* interferendo col processo di allungamento a causa della formazione *in vivo* di una struttura R-loop (ibrido DNA-RNA) a monte dell'espansione GAA, probabilmente per interazione del DNA templato col trascritto nascente, o per interazione con FAST-1, un trascritto antisenso individuato nella stessa regione. In uno studio su *Arabidopsis thaliana*, con inserzione di un'espansione GAA nel gene *IIL1* è stato generato un modello con fenotipo termosensibile che ha permesso di individuare l'accumulo di piccoli RNA interferenti (siRNA) e di repressori in quel locus. Il *knock down* dei componenti del *pathway* di metilazione del

DNA RNA-dipendente ha ristabilito la trascrizione di *IIL1* e ha portato alla scomparsa del fenotipo associato all'espansione ripetuta, suggerendo il coinvolgimento dei siRNA nella patologia, e attribuendo all'espansione GAA un effetto di silenziamento epigenetico (2). L'espressione della proteina fratassina è elevata nei gangli della radice dorsale, nel midollo spinale, nei nuclei dentati cerebellari, nella corteccia cerebrale, nel cuore, fegato e nei muscoli scheletrici, riflettendo i principali siti colpiti dall'FA. La fratassina è coinvolta nella biogenesi dei complessi ferro-zolfo (ISC) nella matrice mitocondriale (3). Gli ISC sono cofattori per proteine del ciclo di Krebs (aconitasi) e della catena respiratoria mitocondriale (complessi I, II e III). Una deficienza di fratassina altera l'attività di questi complessi secondariamente, attraverso il suo effetto sugli ISC, portando a una riduzione della produzione di ATP nel mitocondrio. Inoltre, la deficienza di fratassina porta a ipersensibilità allo stress ossidativo, ad un'alterazione del metabolismo, del trasporto e dello stoccaggio del ferro e all'alterazione del metabolismo lipidico. Le strategie terapeutiche finora valutate mirano ad aumentare i livelli di fratassina o a correggere le disfunzioni a valle della mancanza di fratassina. In ogni caso non esiste ad oggi una terapia che rallenti il decorrere della malattia.

***Drosophila melanogaster* come organismo modello dell'atassia di Friedreich.** *Drosophila melanogaster* è un organismo modello complementare ai modelli mammiferi che rende possibile lo studio di malattie umane. Permette di studiare velocemente *in vivo* il decorrere di malattie genetiche ed individuarne possibili biomarcatori e bersagli farmacologici. Gli studi esistenti in *Drosophila* su FA sfruttano il fenomeno dell'*RNA interference* per portare alla down regolazione del gene *fh* (omologo di *FXN*). Questi modelli permettono di studiare la deficienza di fratassina *in vivo* e in tessuti e cellule specifiche. Tuttavia, i modelli a RNAi presentano svariate limitazioni, a causa dell'efficienza tessuto specifica della tecnica. Ad esempio, il silenziamento del gene tramite RNAi nei neuroni porta ad effetti molto lievi, probabilmente a causa della bassa efficienza del macchinario di interferenza in queste cellule per la mancata sovrespressione di Dicer2. Inoltre, non permettono di testare farmaci che agiscano su *pathways* interferenti con il processo di silenziamento causato dall'espansione GAA. In questo lavoro si è scelto perciò di generare tramite la tecnologia di *genome editing* CRISPR/Cas9 un modello di *Drosophila* il cui silenziamento di *fh* dipendesse dall'espansione GAA, inserendo una porzione del primo introne di *FXN* portante le triplette GAA nell'introne di *fh.* Per lo studio sono state quindi utilizzate delle Drosophile con un'inserzione di 200 GAA, in cui un'analisi Deep Seq ha rivelato un profilo trascrizionale associato alla malattia, utilizzabile come bersaglio per studi farmacologici. Uno *screening* farmacologico ha inoltre portato all'identificazione dell'N-acetil cisteina come molecola "protettrice" al progredire della malattia, aumentando l'aspettativa di vita dei moscerini con deficienza di fratassina.

Approccio sperimentale

L'espansione 200GAA nel gene *fh* riduce l'espressione di fratassina e interferisce con sviluppo larvale e pupale. Il modello *fh*-200GAA è stato prodotto grazie all'utilizzo della tecnologia CRISPR/Cas9: un'espansione GAA di circa 200 triplette, ottenuta tramite PCR dal DNA di un paziente affetto da FA, è stato inserito nell'introne del gene *fh* di *Drosophila* (Fig 1A). L'espansione GAA è fiancheggiata da sequenze del primo introne di FXN (157 nucleotidi a monte e 125 nucleotidi a valle dell'espansione GAA). Il gene *fh* si trova sul cromosoma X di *Drosophila*.

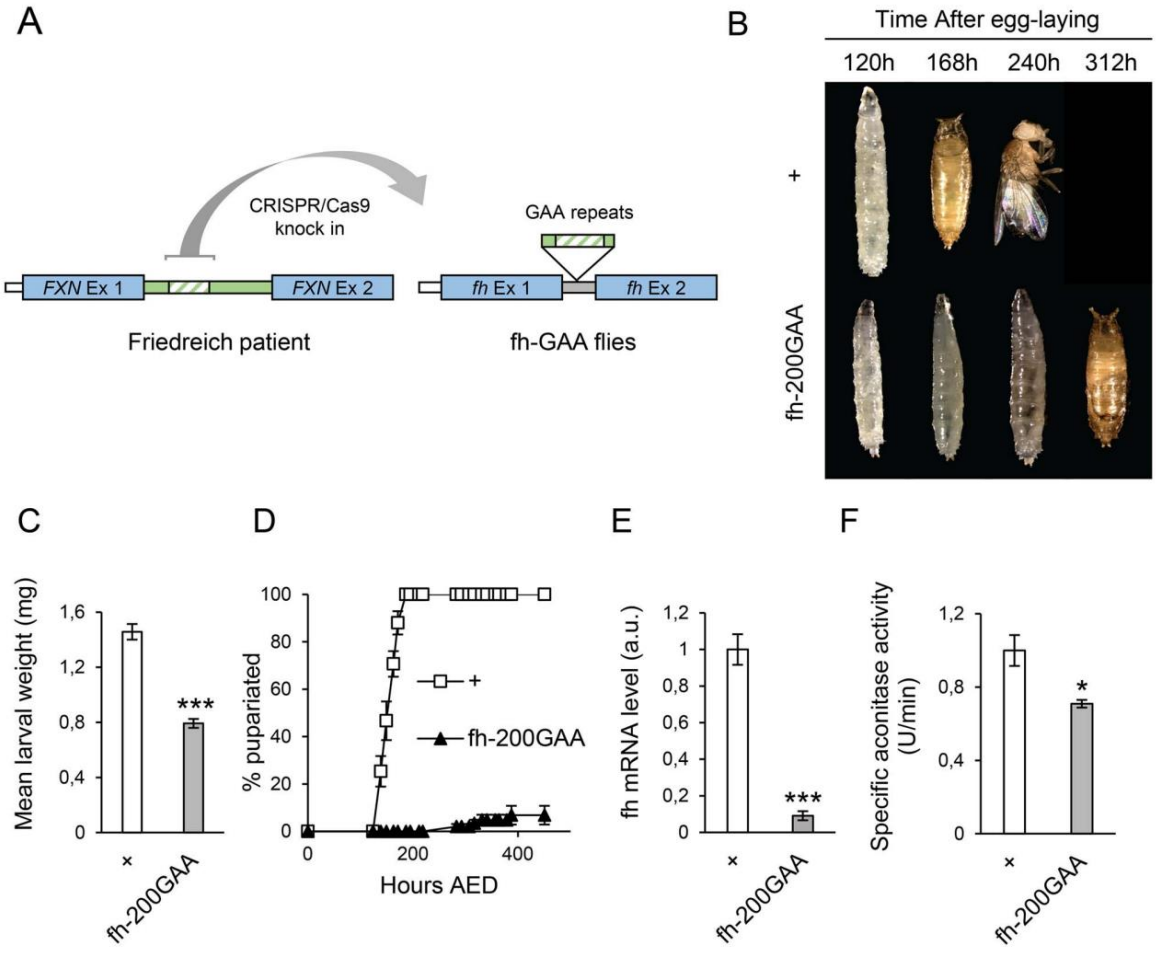

Fig. 1. Generazione dei moscerini *fh*-200GAA e fenotipi dello sviluppo a 26°C. (A) L'espansione 200GAA umana viene inserita nell'introne del gene *fh* di *Drosophila* usando la tecnologia CRISPR/Cas9; (B) Foto di larve, pupe e adulti a diversi intervalli temporali (espressi in ore) dalla deposizione delle uova; (C) Peso delle larve a 144 ore dalla deposizione delle uova; (D) Tempo di pupariazione di 75 campioni di controllo e 146 *fh*-200GAA, espresso come percentuale di raggiungimento individuale dello stadio di pupa nel tempo dalla deposizione delle uova (AED); (E) Quantificazione del trascritto *fh* (qRT-PCR con estratto di mRNA totale da larve a 144 ore dalla deposizione delle uova); (F) Attività aconitasica totale (mitocondriale e citosolica) dei campioni *fh*-200GAA e di controllo (+).

E' stato innanzitutto analizzato l'effetto dell'espansione GAA sullo sviluppo e la vitalità dei moscerini. In condizioni di allevamento standard (26°C) i maschi *fh*-200GAA/Y non hanno raggiunto lo stadio adulto, mentre le femmine eterozigoti *fh*-200GAA/+ erano pienamente vitali. Lo sviluppo di *Drosophila* procede secondo una serie di fasi: embriogenesi, tre stadi larvali, e uno stadio pupale. Le larve maschili hanno subito un ritardo nella crescita (Fig.1B), il loro peso è in media il 46 % del peso di controllo a 144 ore dalla deposizione delle uova (Fig.1C). Sono state poi osservate difficoltà nella fase pupale. Solo il 7 % dei maschi *fh*-200GAA/Y è entrato nello stadio pupale non prima di 250 ore dalla deposizione delle uova (Fig.1D) e nessuno ha completato lo sviluppo ad adulto, mentre il 100 % delle larve di controllo ha raggiunto la fase di pupa in 180 ore. La quantificazione dell'mRNA di *fh* nei maschi *fh*-200GAA/Y ha rivelato una riduzione dell'espressione di fratassina del 91 %

rispetto al controllo (Fig.1E), confermando l'efficacia dell'espansione GAA nel ridurre la quantità di trascritti di fratassina. Anche l'attività aconitasica, ridotta in pazienti umani e altri modelli della malattia compresa *Drosophila* (4), si riduce del 29% nelle larve fh-200GAA/Y (Fig.1F).

Effetto della temperatura sul fenotipo fh-200GAA: a basse temperature si ottengono adulti poco longevi e con difetti motori. A 16 °C i maschi *fh*-200GAA/Y presentano ancora ritardi nella crescita ma in minor misura rispetto alle larve allevate a 26 °C. Il peso medio è inferiore al controllo del 15.5 % (Fig.2A). Anche la pupariazione è ritardata, ma l'89 % delle larve *fh*-200GAA/Y ha formato pupe a 16°C (Fig.2B) e il 16 % ha raggiunto lo stadio adulto, anche se brevemente longeve. Mantenuti a 16°C gradi questi adulti vivono mediamente 10 giorni. Se spostati a 26 °C dopo lo sviluppo ad adulto, la longevità si abbassa a 3 giorni (Fig.2C).

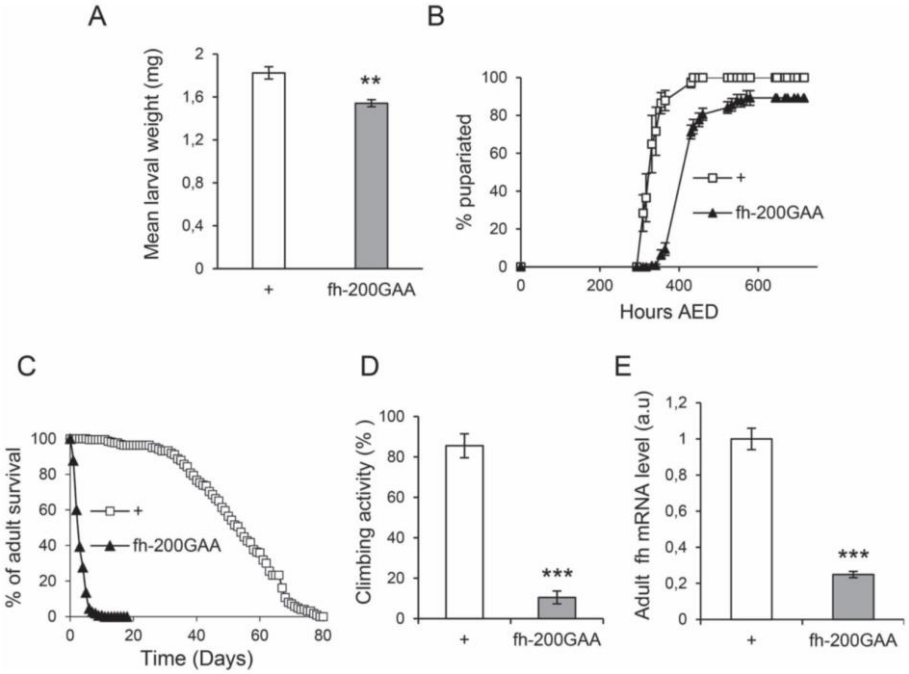

Fig.2. Fenotipi dello sviluppo e dell'adulto in moscerini *fh*-200GAA allevati a 16°C durante lo sviluppo. (A) Peso medio delle larve a 330 ore dalla deposizione delle uova; (B) tempo di pupariazione di 74 campioni di controllo e 139 *fh*-200GAA, espresso come percentuale di raggiungimento individuale dello stadio di pupa nel tempo; (C) Tasso di sopravvivenza di moscerini trasferiti da 16°C a 26°C dopo lo sviluppo ad adulto, misurato come percentuale di sopravvissuti nel tempo, con media di 51 e mediana 53 giorni di vita per i controlli (223 campioni) e di 2,6 e 3 per gli *fh*-200GAA (155 campioni); (D) Saggio di geotassi negativa che ha valutato la capacità di maschi adulti di 1 giorno, sviluppati a 16°C e poi trasferiti a 26°C, di risalire la superfice di un tubo di plastica dopo essere stati fatti cadere sul fondo picchiettando sulle superfici del tubo. Sono stati riportati i risultati superiori a una velocità di risalita di 3 cm in 10 secondi; (E) Quantificazione del trascritto *fh* in moscerini maschi adulti sviluppati da un giorno e trasferiti da 16°C a 26°C.

Anche le capacità locomotorie, valutate in base alla capacità di muoversi verticalmente, appaiono fortemente ridotte conseguentemente al cambio di temperatura (Fig. 2D). Il livello di mRNA di *fh* in

questi maschi adulti allevati a 16°C è inferiore del 75% rispetto ai controlli allevati alla stessa temperatura (Fig.2E). I risultati mostrano che il fenotipo *fh*-200GAA/Y è fortemente influenzato dalla temperatura. Ciò potrebbe essere dovuto a una minore necessità di fratassina a basse temperature in conseguenza di un rallentamento metabolico, oppure ad una più diretta influenza della temperatura sull'effetto silenziatore dell'espansione GAA. La quantificazione dell'mRNA di *fh* in larve 200GAA allevate a 20°C e a 26°C, e nei rispettivi controlli, ha rivelato che l'espressione di *fh* nei controlli è inferiore a 20°C rispetto a 26°C e che la temperatura invece non modifica statisticamente la quantità di trascritti nelle larve *fh*-200GAA (dato non mostrato). Tutto ciò suggerisce che l'influenza della temperatura sulla forza del fenotipo sia dovuta a una minor necessità di fratassina a basse temperature in risposta a una minore richiesta metabolica.

La gravità del fenotipo è associata alla dimensione dell'espansione GAA: fenotipo fh-18GAA. Per studiare la forza del fenotipo in funzione della lunghezza dell'espansione GAA, è stata generata una linea di *Drosophila* con un'espansione di solo 18 triplette. A 26°C questa linea è vitale, a differenza della linea *fh*-200GAA allevata alla stessa temperatura, ma con una durata di vita media di solo 7,4 giorni. A 16°C questo valore sale a 44 giorni per gli *fh*-18GAA, mentre per gli *fh*-200GAA a questa temperatura era di solo 10 giorni, mostrando che la dimensione dell'espansione riduce l'effetto del fenotipo sulla vitalità e sulla longevità (tabella 1).

	fh-200GAA		*fh-18GAA*		*w1118* control		ratio Median lifespan
Temperature	Median lifespan	n	Median lifespan	n	Median lifespan	n	*fh-18GAA/w1118*
16°C	**10,3**	39	**43,8**	168	ND		ND
20°C	Not viable		**30**	180	**106**	171	**0,28**
23°C	Not viable		**16,2**	164	**60**	161	**0,27**
26°C	Not viable		**7,4**	181	**44,7**	164	**0,17**

Tabella 1. Durata di vita media di *Drosophile fh*-18GAA, *fh*-200GAA e controllo a varie temperature, indicata in giorni. n = numero di moscerini. ND: non determinato.

La relazione di proporzionalità inversa tra temperatura e durata del ciclo vitale di *Drosophila* è nota; perciò, per dimostrare la dipendenza del fenotipo *fh*-18GAA dalla temperatura, sono stati calcolati i rapporti tra la durata della vita media degli *fh*-18GAA e dei controlli a diverse temperature. A 26°C il rapporto è di 0.17, a 23°C è 0.26 e 0.28 a 20°C, evidenziando una maggior longevità del fenotipo 18GAA col diminuire della temperatura (tabella 1).

In ogni caso, l'espansione di 18GAA è di carattere patologico in *Drosophila*. Il livello di mRNA di *fh* nelle larve *fh*-18GAA è infatti ridotto rispetto al controllo, anche se in proporzione minore rispetto agli individui *fh*-200GAA alle stesse temperature (dato non mostrato). Ciò suggerisce una maggior suscettibilità all'espansione GAA nei moscerini piuttosto che nell'uomo, anche se non si può escludere che l'alterazione nell' espressione di fratassina sia influenzata dalla porzione di sequenza intronica umana inserita nell'introne di *fh*.

Induzione dell'espressione di fratassina per ovviare alla letalità dell'espansione 200GAA. Si è poi scelto di utilizzare il sistema inducibile GS (*Gene Switch*) per verificare se l'incremento dell'espressione di *fh* fosse sufficiente a correggere il fenotipo 200GAA. Il sistema consiste in due

transgeni: daGS, con la sequenza per la proteina Gene Switch (GS) ubiquitariamente espressa sotto in controllo del promotore *daughterless* (da), e UAS-*fh*, in cui l'espressione di *fh* è mediata dal riconoscimento dell'*upstream regulatory sequence* (UAS) da parte della proteina GS dopo interazione con RU486 (fornito nell'alimentazione) (FIG.3A).

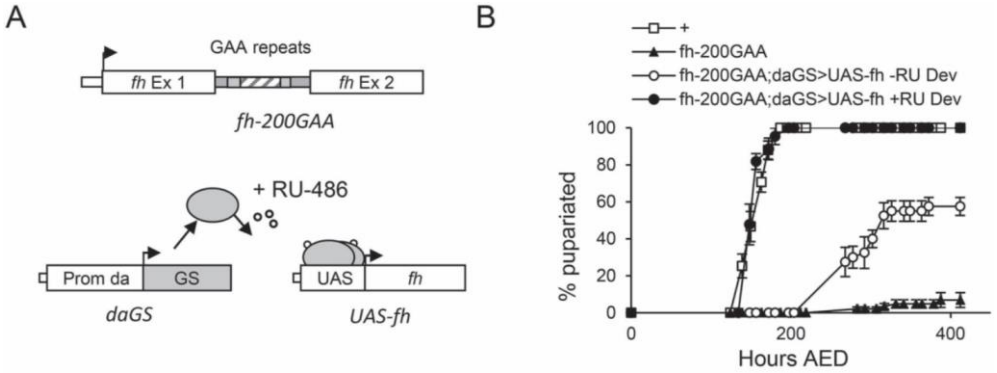

Fig.3 A e B. Sistema d'induzione e efficacia. Espressione di *fh* sotto il controllo del sistema inducibile GS (A); tempo di pupariazione, espresso come percentuale di raggiungimento individuale dello stadio di pupa nel tempo (B).

Ciò permette di far esprimere la fratassina ubiquitariamente solo se RU486 è presente nel cibo. Senza RU486 le larve non hanno raggiunto lo stadio adulto, e presentano ritardi e difetti allo stadio pupale. Il 57% delle larve *fh*-200GAA/Y; daGS>UAS-*fh* ha raggiunto lo stadio di pupa contro il 7% delle *fh*-200GAA/Y, probabilmente a causa di un'attiva trascrizione basale tessuto specifica del gene UAS-*fh* anche in mancanza di RU486, che però non è stata rilevata dall'analisi di qPCR su estratto di larve intere (Fig. 3B).

RU486, se fornito durante lo sviluppo, permette di ottenere adulti sani senza alcun ritardo. A sviluppo terminato i maschi *fh*-200GAA/Y; daGS>UAS-*fh* vengono perciò divisi in due gruppi: trattati con RU486, che presentano una longevità simile ai wild-type (33 giorni), e non trattati con RU486, con vita media di 11.5 giorni ed evidenti difetti locomotori (Fig.3C-D), dimostrando che il fenotipo *fh*-200GAA è causato dalla deplezione di fratassina. Si è applicato lo stesso procedimento con Drosophile *fh*-18GAA; daGS>UAS-*fh*, osservando ancora una volta effetti patologici più deboli sui campioni 18GAA piuttosto che sui 200GAA (dato non mostrato). I campioni non trattati con RU486 dopo l'entrata in fase adulta presentano una durata di vita di 29 giorni senza effetti sulle capacità di movimento, mentre quelli trattati anche in fase adulta hanno raggiunto una media di 36 giorni (dato non mostrato), confermando che la ridotta longevità degli esemplari 18GAA è dovuta a una deficienza di fratassina.

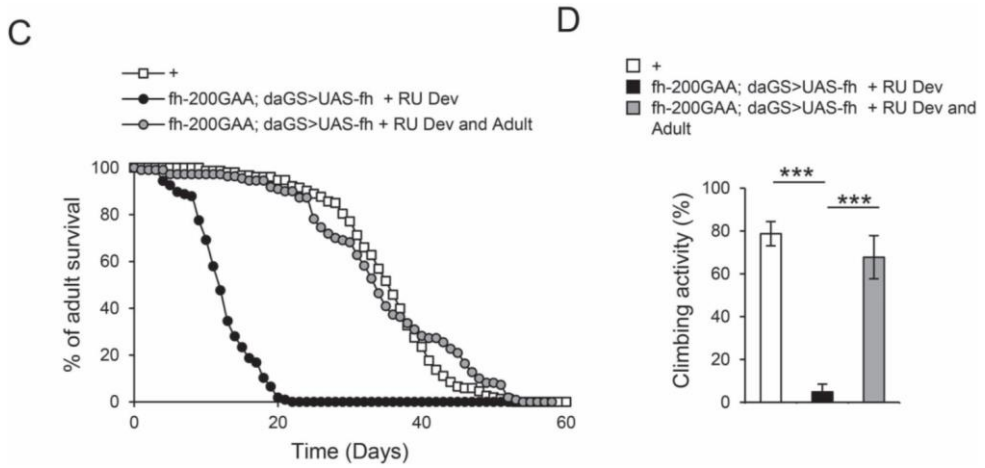

Fig.3 C e D. Effetti dell'induzione di UAS-fh. (C) Curva di sopravvivenza, espressa come percentuale di maschi sopravvissuti nel tempo, trattati con RU486 (10 μg/ml) durante lo sviluppo e poi divisi in due gruppi, trattati (100 μg/ml) e non trattati. Il controllo (+) ha continuato il trattamento nella fase adulta a 10 μg/ml; (D) Saggio di geotassi negativa, espressa come percentuale di moscerini di 8 giorni in grado di risalire più di 5 cm in 10 secondi. Tutti gli esperimenti sono stati condotti a 26°C.

Analisi del trascrittoma dei moscerini con deficienza di fratassina: analisi dell'espressione genica differenziale e di *Gene Ontology*. Per ottenere un'ampia panoramica delle modificazioni d'espressione genica causate dalla deficienza di fratassina, il trascrittoma degli *fh*-200GAA/Y; daGS>UAS-*fh* trattati con RU486 solo durante lo sviluppo è stato analizzato e paragonato a quello di campioni che hanno continuato il trattamento. La quantificazione dei trascritti *fh* tramite qRT-PCR ha mostrato che i campioni (raccolti a 4, 8 e 12 giorni dall'entrata in fase adulta), se non trattati con RU486 mostrano una diminuzione della trascrizione di *fh* del 40% rispetto al controllo (Fig.4A).

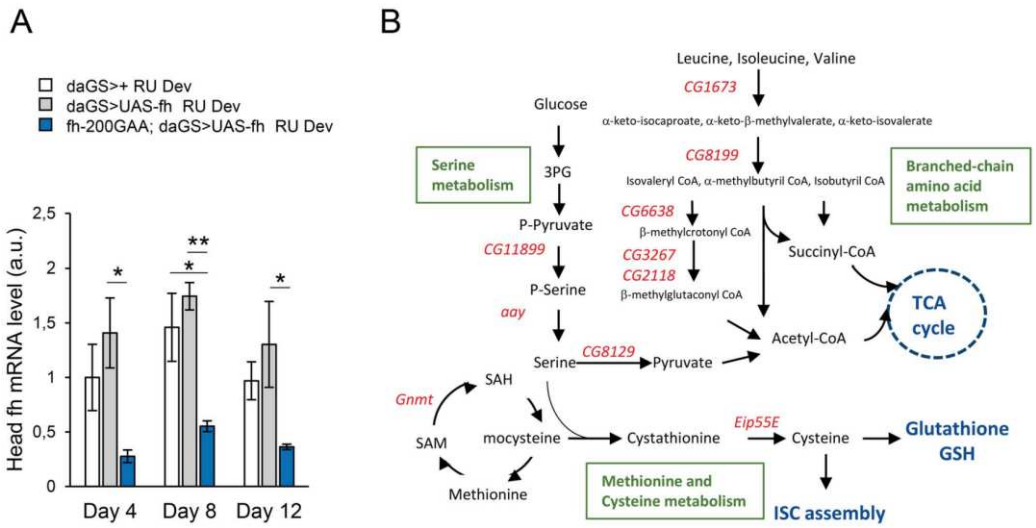

Fig.4 A e B. Analisi del trascrittoma dei moscerini con deficienza di fratassina. (A) Quantificazione del trascritto *fh* a 4, 8 e 12 giorni in campioni adulti trattati con RU486 solo durante lo sviluppo. (B) Schema dei geni *up-regolati* nel metabolismo degli amminoacidi (geni in rosso) in condizioni di deficienza di fratassina.

Si è svolta poi su individui con deficienza di fratassina con 5 giorni di vita un'analisi di RNA Seq, che ha permesso di individuare i cambiamenti trascrizionali precoci alla base della malattia. Per limiti di spazio, in questo elaborato non vengono riportate le tabelle con i risultati di queste analisi. Sono stati identificati 230 geni *up-regolati* e 88 *down-regolati* in condizioni di deficienza di fratassina. L'analisi di *Gene Ontology* svolta sulla piattaforma PANTHER ha raggruppato nel set di geni *up-regolati* 42 geni codificanti proteine mitocondriali, evidenziando un forte arricchimento in questo compartimento subcellulare.

L'arricchimento in funzioni molecolari si ha nelle categorie di cofattori di *binding* (30 geni sovrespressi), attività catalitica (130 geni sovrespressi) e di ossidoreduttasi (49 geni sovrespressi). In particolare, nella categoria delle ossidoreduttasi appaiono due geni per la ferredossina in *Drosophila* (*Fdxh* e *Fdx2*), la cui sovrespressione potrebbe essere interpretata come un tentativo di aumentare la velocità di sintesi degli ISC: *FDX2*, che codifica la ferredossina nei mammiferi, gioca infatti un ruolo essenziale nella sintesi dei complessi Fe-S. Inoltre, appare sovrespresso *Nmdmc* (un gene coinvolto nel *pathway* di biosintesi delle purine) notoriamente attivato in condizioni di stress ossidativo in Drosophila (5). Anche l'attività della GST (Glutatione-S-transferasi), un enzima evolutivamente conservato con attività detossificante essenziale alla cellula per proteggersi da danni ossidativi (6), risulta arricchita con 11 geni *up-regolati* nei campioni con deficienza di fratassina.

Gli arricchimenti in processi biologici si concentrano, in accordo con gli arricchimenti in funzioni molecolari, sui processi di ossidoriduzione, sul metabolismo del glutatione e sui *pathway* del metabolismo degli amminoacidi (FIG.4B). Due geni risultano coinvolti nel metabolismo della metionina e della cisteina: *Gnmt* e *Eip55E*. Il primo codifica per un enzima che catalizza la conversione di S-adenosilmetionina in S-adenosilomocisteina e il secondo per una cistationina gamma-ligasi coinvolta nella biosintesi della cisteina. L'*up-regolazione* di questi geni può essere interpretata come un tentativo di aumentare il *pool* generale di cisteina per aumentare il livello di glutatione (derivato dalla cisteina) o per aumentare la velocità di sintesi degli ISC. Inoltre, si trovano arricchimenti di sovrespressione nel catabolismo degli amminoacidi ramificati (BCAA) che sfocia nella produzione di Acetil-CoA e Succinil-CoA, e nel metabolismo della serina, in particolare in geni codificanti enzimi che convertono serina e treonina in piruvato.

L'insieme di geni *up-regolati* suggerisce che la risposta trascrizionale indotta dalla deficienza di fratassina porti ad un potenziamento della risposta a stress ossidativo e della biosintesi degli ISC, e al tentativo di ricostituire gli intermedi del ciclo degli acidi tricarbossilici (TCA) (Fig.4B). Il set di geni *down-regolati* è più limitato. L'arricchimento in componenti cellulari ha evidenziato la categoria extracellulare. In particolare, tra questi geni è presente *Tsf1* codificante la transferrina, una proteina secretoria coinvolta nel trasporto del ferro, la cui repressione è stata precedentemente dimostrata in conseguenza ad accumulo di ferro.

Identificazione di Tspo come biomarcatore dell'atassia di Friedreich. Il gene *Tspo* è compreso nella categoria di quelli *up-regolati* nell'analisi Deep-Seq, ed è particolarmente attraente come *biomarker* putativo per la deficienza di fratassina in Drosophila: è stato infatti mostrato che nell'uomo l'espressione di TSPO è aumentata in diverse malattie neurodegenerative ed è già stato proposto come *biomarker* di danni cerebrali e infiammazione neuronale (7). TSPO codifica una proteina evolutivamente conservata localizzata nella membrana mitocondriale esterna, coinvolta in svariate

funzioni mitocondriali come il trasporto del colesterolo, la biosintesi degli ormoni steroidei e dell'eme, l'apoptosi e la proliferazione cellulare (8). I campioni derivanti dalle teste degli esemplari *fh*-200GAA/Y daGS>UAS-*fh* allevate a 26°C, trattate con RU486 solo durante lo sviluppo e con deficienza di fratassina nell'adulto, presentano un progressivo aumento nell'espressione di *Tspo*, riportato alla normalità dall'espressione di fratassina nell'adulto (Fig.4D). Di conseguenza, l'espressione di *Tspo* può essere usata come marcatore molecolare del progredire del fenotipo indotto dalla deficienza di fratassina. Il tentativo di aumentare o silenziare l'espressione di *Tspo* tramite interferenza a RNA nelle larve fh-200GAA non ha in nessun caso risolto il problema della letalità nello sviluppo.

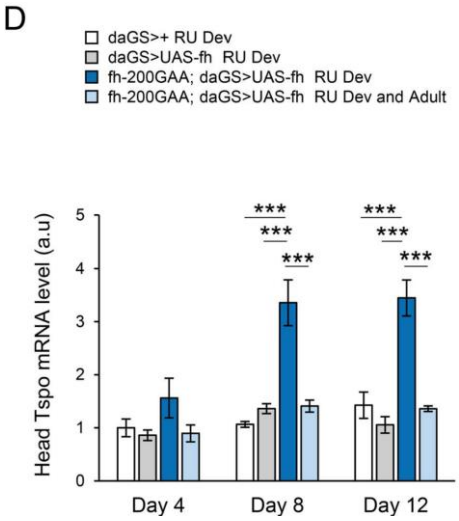

Fig.4D. Analisi del trascrittoma dei moscerini con deficienza di fratassina. (D) Quantificazione del trascritto *Tspo* tramite qRT-PCR da estratto di RNA totale di teste di maschi adulti di 4, 8 e 12 giorni. Tutti i campioni sono stati trattati con RU486 durante lo sviluppo e poi divisi in trattati e non trattati durante la fase adulta.

Effetto positivo del trattamento con N-Acetil cisteina. Si è infine scelto di testare l'efficacia farmacologica dell'N-acetil Cisteina (NAC) a diverse concentrazioni. I campioni trattati con 200 µg/ml di NAC nel cibo non hanno mostrato un aumento sulla longevità. Il trattamento a concentrazioni più elevate, 400 e 800 µg/ml, ha portato rispettivamente ad un incremento della longevità del 13 e 35% evidenziando così un effetto dose dipendente (Fig.5A). Se i campioni vengono trattati sia durante lo sviluppo che durante la fase adulta, la sopravvivenza aumenta al 47%, evidenziando una maggior efficacia se il trattamento avviene prima della fase adulta (Fig.5B). Anche le capacità locomotorie appaiono migliorate sia nei campioni costantemente trattati, che in quelli trattati solamente allo stadio adulto (Fig.5C). Il trattamento con NAC aumenta significativamente l'attività aconitasica delle larve *fh*-200GAA (Fig.5F).

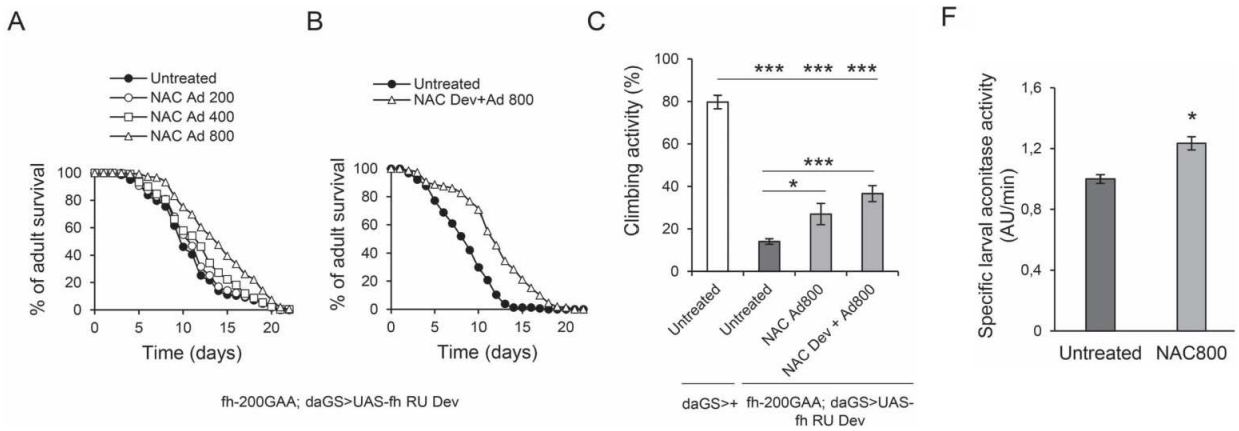

Fig.5. Effetto protettivo del trattamento con NAC. (A) Curva di sopravvivenza di adulti *fh*-200GAA;daGs>UAS-fh trattati con diverse concentrazioni di NAC (NAC=200 μg/ml, n = 282; NAC=400 μg/ml, n = 242; NAC=800 μg/ml, n = 149; non trattati, n = 143). (B) curva di sopravvivenza di moscerini *fh*-200GAA;daGs>UAS-fh non trattati con NAC e trattati con 800 μg/l durante sviluppo e fase adulta. L'aumento in longevità è del 46 %. (C) Saggio di geotassi negativa effettuato su moscerini *fh*-200GAA;daGs>UAS-fh sottoposti a trattamento con NAC (800 μg/l) durante tutto il ciclo vitale oppure solo durante lo sviluppo. Tutti i campioni sono sempre stati trattati con RU486 (10 μg/ml) durante lo sviluppo. (D) Attività aconitasica totale (citosolica e mitocondriale) di larve *fh*-200GAA trattate e non trattate con NAC.

Discussione

Simulazione della malattia in *Drosophila melanogaster* e meccanismo molecolare del silenziamento GAA: Interpretazione dei risultati. Nel lavoro presentato in questo elaborato di laurea, è stato messo a punto e caratterizzato un nuovo modello di *Drosophila* per lo studio dell'atassia di Friedreich (FA) mediante l'inserzione dell'espansione GAA nel gene *fh*, omologo di *FXN*. Il modello *fh*-200GAA riproduce numerose caratteristiche della malattia umana, inclusi i diminuiti livelli di espressione di fratassina, una ridotta durata di vita e una disfunzione locomotoria. Il livello dei trascritti di fratassina nei maschi emizigoti *fh*-200GAA/Y è inferiore del 75-90% rispetto al normale, in accordo coi risultati ottenuti da studi sull'espressione di FXN in pazienti umani affetti da FA (9). Per ciò che concerne il meccanismo di silenziamento indotto dall'espansione GAA, l'analisi DeepSeq non ha individuato significative diminuzioni nei due geni adiacenti al locus *fh* (*Bap111* e *CG7065*, entrambi localizzati a meno di 1 kb dall'espansione GAA), suggerendo che se il silenziamento è di natura epigenetica, questo è limitato a una piccolissima regione. Inoltre, la minor intensità del fenotipo patologico nei campioni 18GAA, avvalora l'ipotesi di un impedimento trascrizionale nel processo di allungamento, dipendente appunto dalla lunghezza dell'espansione. L'insorgere di effetti patologici, anche se minori, negli individui *fh*-18GAA (quindi con un'espansione più ridotta) dimostra una maggior sensibilità all'espansione GAA in *Drosophila* piuttosto che nei mammiferi. Invece, per quanto riguarda l'instabilità mitotica e meiotica dell'espansione GAA dimostrata nei mammiferi, non sono state rilevate tramite analisi PCR su DNA genomico alterazioni dal punto di vista della lunghezza dell'espansione e del fenotipo annesso negli esemplari utilizzati (derivanti da una linea di circa 50 generazioni), suggerendo questa caratteristica di stabilità come tipica di Drosophila. Ciò rende questo organismo modello molto attraente per effettuare studi su meccanismi patologici ed eventuali interventi farmacologici con un'elevatissima riproducibilità nel

tempo. Questo studio ha inoltre messo in evidenza le potenzialità di *Drosophila* come modello per lo studio di malattie causate da espansioni trinucleotidiche (TNR).

Analisi di espressione genica differenziale: interpretazione dei risultati. L'analisi dei geni differenzialmente espressi, nei campioni in cui l'espressione di fratassina è stata indotta solo durante lo sviluppo, ha rivelato arricchimenti che suggeriscono la forte attivazione dei meccanismi di protezione da stress ossidativo (GST, CYP450 e perossidasi). L'*up-regolazione* riscontrata nei geni della ferredossina potrebbe essere una conseguenza dell'innesco di un *loop* trascrizionale di regolazione per far fronte alla diminuzione della velocità di biosintesi degli ISC (*Iron Sulfur Clusters*). È stato individuato un arricchimento di sovrespressione nei geni partecipanti al catabolismo degli amminoacidi ramificati (BCAA). Curiosamente, un'elevata attività del catabolismo dei BCAA è stata rilevata in pazienti con malattia di Huntington per compensare deficit energetici nel cervello (10). Nel lavoro analizzato nell'elaborato l'effetto della deficienza di fratassina sul catabolismo dei BCAA non è stato delucidato, ma meriterebbe ulteriori attenzioni.

È stata individuata anche una forte e progressiva attivazione dell'espressione di *Tspo* nei campioni con deficienza di fratassina. Nei mammiferi Tspo è principalmente presente nel tessuto adiposo con una bassa espressione nel sistema nervoso, confinato solamente nelle cellule gliali e in alcuni particolari tipi neuronali. L'espressione neuronale di TSPO è stata associata, in studi su malattie neurodegenerative (Alzheimer, sclerosi laterale amiotrofica, Parkinson, ecc) alla presenza di danni neuronali. Inoltre, i risultati degli studi su pazienti affetti da FA hanno evidenziato una sovrespressione di TSPO in campioni di sangue periferico, avvalorando la scelta di Tspo come *biomarker* per la malattia.

Trattamento farmacologico con NAC: potenziali meccanismi d'azione dell'N-Acetil cisteina. Il trattamento con N-acetil cisteina ad elevate concentrazioni ha evidenziato un effetto di protezione, aumentando la percentuale di sopravvivenza dei campioni trattati. L'N-acetil cisteina è una molecola dalla nota azione antiossidante che può essere esplicata direttamente come composto tiolico o indirettamente agendo da fonte di cisteina per aumentare il *pool* cellulare di glutatione. Inoltre, l'N-acetil cisteina incrementa l'attività dell'aconitasi (un enzima con Fe-S), suggerendo che possa agire indirettamente da antiossidante anche prevenendo la distruzione dei ISC. Nel contesto di deficienza di fratassina, l'*up-regolazione* dei geni per la sintesi di cisteina (*Gnmt* e *Eip55E*) avvalora l'ipotesi secondo cui l'NAC potrebbe anche fungere direttamente da fonte di cisteina, o da donatore di zolfo per la biosintesi degli ISC. Questo studio evidenzia le potenzialità terapeutiche di questo composto per stimolare successive indagini cliniche.

Materiali e Metodi

Linee di *Drosophila* e metodi di coltura. Per generare la linea di *Drosophila* fh-GAA, l'espansione GAA, fiancheggiata da sequenze introniche di *FXN*, è stata amplificata tramite PCR dal DNA genomico di un paziente affetto da FA, utilizzando i seguenti *primers*: 5'-AATGGATTTCCTGGCAGGACGC-3' e 5'-GCATTGGGCGATCTTGGCTTAA-3'. Il prodotto di PCR è stato inserito nel plasmide PUC19, 991 bp a valle e 995 bp a monte del locus *fh*, costituendo la regione di omologia del plasmide donatore, che ha permesso poi l'inserzione della sequenza umana tra la posizione 13 e 37 dell'introne di *fh*. Il plasmide donatore per i costrutti fh-18GAA è stato ottenuto

per contrazione dell'espansione GAA nel ceppo NEB5-α *E. coli*. Il plasmide donatore e il gRNA (RNA guida) sono stati iniettati in una linea yw;attP40(nos-Cas9)/CyO, che è stata successivamente incrociata con linee UAS-fh, FM7i e FM7tb per bilanciare il cromosoma X fh-GAA, e ulteriormente incrociate con una linea daGS. Il cibo utilizzato in tutti gli esperimenti contiene 82.5 mg/ml di lievito, 34 mg/ml di farina di mais, 50 mg/ml di saccarosio, 11.5 mg/ml agar, 27.8 µl/ml di metil 4-idrossibenzoato. Quando richiesto dall'esperimento, è stato aggiunto RU486 da una soluzione *stock* in etanolo 20mg/ml.

RNA-Seq e analisi dell'espressione genica differenziale. L'analisi dei geni differenzialmente espressi parte da estratto di RNA totale da teste maschili di 5 giorni trattate con RU486 (10 µg/ml) solo durante lo sviluppo. Per l'analisi di RNA-seq sono state inviate a IntraGen (Genopole, Evry, Francia) tre repliche per ogni genotipo. Il c-DNA è stato sintetizzato tramire kit Illumina HiSeq400. La qualità delle letture per ogni campione è stata valutata con FastQC. La mappatura sul genoma di Drosophila (BDGP6) è stata eseguita con STAR (V.2.5.). Il numero di letture mappate per ogni gene è stato calcolato con "HTSeq count" e utilizzato per calcolare il valore FPKM di ogni gene. Per l'analisi dei geni differenzialmente espressi è stato usato il pacchetto di R DESeq2. Gli arricchimenti di Gene ontology (GO) sono stati calcolati con PHANTER.

Quantificazione dei trascritti mediante qRT-PCR. Per la quantificazione dei trascritti tramite qRT-PCR l'RNA è stato estratto col kit PicoPureTM RNA (Thermo Fisher Scientific). I cDNA sono stati sintetizzati da RNA totale isolato usando la retrotrascrittasi SusperScript™III (Thermo Fisher Scientific). La qRT-PCR è stata condotta col qPCR Mix (Promega) su un LightCycler480 (Roche). Per la normalizzazione è stato usato come riferimento il gene ribosomale rp49. I *primers* utilizzati per l'amplificazione sono i seguenti: 5'-ACACCCTGGACGCACTGT-3' and 5'-CCAGGTTCACGGTTAGCAC-3' per il gene *fh*, 5'-TCGGGCAGCACAACATCAAG-3' e 5'-GTACGAAGAGCAATCCAGCG-3' per il gene *Tspo* e 5'-CCGCTTCAAGGGACAGTATCT-3' e 5'-CACGTTGTGCACCAGGAACTT-3' per il gene *rp49*. La quantificazione di ogni campione è stata effettuata su 5 repliche.

Esperimenti di *lifespan* e trattamento con NAC. I moscerini sono stati catturati a 24 ore dalla schiusa delle uova previa anestesia con CO_2. Sono stati allevati in gabbie da 30 individui a 26°C con 12 ore di luce e 12 di buio. I moscerini sono stati trasferiti in terreno fresco ogni 2 giorni. NAC (Sigma Aldrich) è stata aggiunta al cibo a 37°C da una soluzione di stoccaggio (80mg/ml).

Bibliografia

1- Santos, R., Lefevre, S., Sliwa, D., Seguin, A., Camadro, J.M. and Lesuisse, E. (2010) Friedreich ataxia: molecular mechanisms, redox considerations, and therapeutic opportunities. Antioxid. Redox Signal., 13, 651–690.
2- Friedreich Ataxia – pathogenesis and implications for therapies (Martin B. Delatycki, Sanjay I. Bidichandani)
3- Braymer, J.J. and Lill, R. (2017) Iron-sulfur cluster biogenesis and trafficking in mitochondria. J. Biol. Chem., 292, 12754–12763.
4- Llorens, J.V., Navarro, J.A., Martinez-Sebastian, M.J., Baylies, M.K., Schneuwly, S., Botella, J.A. and Molto, M.D. (2007) Causative role of oxidative stress in a Drosophila model of Friedreich ataxia. FASEB J., 21, 333–344.
5- Yu, S., Jang, Y., Paik, D., Lee, E. and Park, J.J. (2015) Nmdmc overexpression extends Drosophila lifespan and reduces levels of mitochondrial reactive oxygen species. Biochem. Biophys. Res. Commun., 465, 845–850.
6- Saisawang, C., Wongsantichon, J. and Ketterman, A.J. (2012) A preliminary characterization of the cytosolic glutathione transferase proteome from Drosophila melanogaster. Biochem. J., 442, 181–190
7- Guilarte, T.R. (2019) TSPO in diverse CNS pathologies and psychiatric disease: a critical review and a way forward. Pharmacol. Ther., 194, 44–58.
8- Bonsack, F. and Sukumari-Ramesh, S. (2018) TSPO: an evolutionarily conserved protein with elusive functions. Int. J. Mol. Sci., 19, 1694
9- Li, Y., Lu, Y., Polak, U., Lin, K., Shen, J., Farmer, J., Seyer, L., Bhalla, A.D., Rozwadowska, N., Lynch, D.R. et al. (2015) Expanded GAA repeats impede transcription elongation through the FXN gene and induce transcriptional silencing that is restricted to the FXN locus. Hum. Mol. Genet., 24, 6932–6943
10- Andersen, J.V., Skotte, N.H., Aldana, B.I., Norremolle, A. and Waagepetersen, H.S. (2019) Enhanced cerebral branchedchain amino acid metabolism in R6/2 mouse model of Huntington's disease. Cell. Mol. Life Sci., 76, 2449–2

Appendice:

L'articolo discusso nel presente elaborato è riportato di seguito.

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General Article

GENERAL ARTICLE

A Drosophila model of Friedreich ataxia with CRISPR/Cas9 insertion of GAA repeats in the frataxin gene reveals *in vivo* protection by *N*-acetyl cysteine

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Abstract

Friedreich ataxia (FA) is caused by GAA repeat expansions in the first intron of *FXN*, the gene encoding frataxin, which results in decreased gene expression. Thanks to the high degree of frataxin conservation, the *Drosophila melanogaster* fruitfly appears as an adequate animal model to study this disease and to evaluate therapeutic interventions. Here, we generated a Drosophila model of FA with CRISPR/Cas9 insertion of approximately 200 GAA in the intron of the fly frataxin gene *fh*. These flies exhibit a developmental delay and lethality associated with decreased frataxin expression. We were able to bypass preadult lethality using genetic tools to overexpress frataxin only during the developmental period. These frataxin-deficient adults are short-lived and present strong locomotor defects. RNA-Seq analysis identified deregulation of genes involved in amino-acid metabolism and transcriptomic signatures of oxidative stress. In particular, we observed a progressive increase of *Tspo* expression, fully rescued by adult frataxin expression. Thus, *Tspo* expression constitutes a molecular marker of the disease progression in our fly model and might be of interest in other animal models or in patients. Finally, in a candidate drug screening, we observed that *N*-acetyl cysteine improved the survival, locomotor function, resistance to oxidative stress and aconitase activity of frataxin-deficient flies. Therefore, our model provides the opportunity to elucidate *in vivo*, the protective mechanisms of this molecule of therapeutic potential. This study also highlights the strength of the CRISPR/Cas9 technology to introduce human mutations in endogenous orthologous genes, leading to Drosophila models of human diseases with improved physiological relevance.

Introduction

Friedreich ataxia (FA, OMIM#229300), the most common recessive ataxia, is a progressive neurodegenerative disease caused by mutations in *FXN*, the gene encoding the mitochondrial protein frataxin. Most patients (96%) are homozygous for expanded GAA triplet-repeat sequences located in the first intron of *FXN* that decrease transcription and consequently reduce the levels of the frataxin protein to 5–30% of the normal level (1). The main neurological features are progressive ataxia, sensory loss, pyramidal signs, tendon areflexia and dysarthria (2–4). The first symptoms usually appear before the age of 25 years, with patients losing the ability to walk usually 10–15 years after the onset of the disease and the life expectancy of patients is of approximately 37 years

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(5). Frataxin is ubiquitously expressed, and reduced levels of the protein also affect non-neuronal tissues. In particular, most FA patients develop a hypertrophic cardiomyopathy, which is the leading cause of death (6).

Frataxin is involved in the mitochondrial biogenesis of iron–sulfur clusters (ISC), which are essential protein cofactors involved in a large number of cellular functions (7). This probably explains why frataxin deficiency leads to a very large panel of cellular dysfunctions in patients and in animal or cellular models of the disease, including decreases in aconitase and mitochondrial respiratory chain activities, hypersensitivity to oxidative stress, alterations of iron metabolism and dyshomeostasis of lipid metabolism (8,9). Several clinical trials have been or are currently conducted to evaluate pharmacological treatments in FA patients. The therapeutic strategies aim either to increase frataxin level or to correct cellular dysfunctions induced by frataxin deficiency, for instance by lowering oxidative stress, improving mitochondrial function or reducing iron-mediated toxicity (10). However, there is currently no treatment with proven efficacy to slow down the progression of the disease.

The fruitfly *Drosophila melanogaster* has emerged as an attractive complementary animal model to mammals for studying human diseases (11). It allows fast *in vivo* studies to better understand the development and progression of genetic diseases, to identify genes that might be relevant biomarkers or pharmacological targets and to perform pharmacological screens. Drosophila models of FA are currently mainly based on down-regulation of *fh*, the Drosophila frataxin homolog, by RNA interference (12–18). A missense mutant allele of *fh* was also recently identified, leading to a strong loss of function associated with developmental arrest in larval stages (19). These models already led to the identification of pathological mechanisms and of several drugs partially overcoming frataxin deficiency (reviewed in 20). RNAi-based FA models are particularly interesting in that they allow to study *in vivo* the effects of frataxin deficiency specifically in tissues or cell types relevant for the disease, in particular glial cells and cardiomyocytes (13,15,17,18). However, these RNAi-based models have several limitations: first, frataxin inactivation in neurons led to very mild effects, probably due to the low efficiency of the RNAi machinery in these cells in the absence of Dicer2 overexpression (21). Second, they do not allow to test drugs or pathways that aim to interfere with GAA-induced silencing mechanisms. For these reasons, we decided to use the powerful CRISPR/Cas9 genome editing technology to develop a novel Drosophila model of FA, called fh-200GAA, in which a portion of the first intron of the human *FXN* gene carrying a GAA triplet expansion is inserted in the *fh* intron.

Here, we present the characterization of this model, which reproduces the main features of the FA disease. We performed Deep Seq analysis on these fh-200GAA flies and identified transcriptomic signatures of frataxin-deficiency that can potentially be used as molecular markers in pharmacological tests. Finally, we tested candidate drugs and identified *N*-acetyl cysteine (NAC) as a protective molecule increasing the survival of frataxin-deficient flies.

Results

A 200 GAA expansion in the frataxin gene decreases frataxin expression and affects larval and pupal development

We generated the fh-200GAA model using the CRISPR/Cas9 technology: a GAA expansion of about 200 units, obtained by PCR on genomic DNA of a FA patient, was inserted in the intron of the Drosophila *fh* gene (Fig. 1A). This GAA expansion is flanked by sequences of the human first intron of *FXN* (157 nucleotides upstream and 125 nucleotides downstream of the GAA expansion). We first analyzed the effect of the GAA expansion on fly development and viability. The *fh* gene is located on chromosome X. Under standard husbandry conditions (room temperature or 26°C), *fh-200GAA/Y* males did not reach the adult stage, whereas *fh-200GAA/+* heterozygote females were fully viable. Drosophila development proceeds through a series of stages: embryogenesis, three larval stages, and a pupal stage. At 26°C, fh-200GAA third instar male larvae exhibited delayed growth (Fig. 1B). Their mean weight was 46% lower compared to controls of the same age, 144 h after egg laying (Fig. 1C). We also observed defective pupariation: when 100% of control larvae reached the pupal stage at 180 h; the first fh-200GAA male pupae were not observed before 250 h after egg laying. Only 7% finally reached the pupal stage (Fig. 1D) but all failed to give adults at this temperature. Some larvae remained alive for up to 13 days of development and continued to feed and grow but without reaching the final weight of control larvae entering the pupal stage. A similar retarded development and extended third larval stage were previously observed with RNAi-mediated ubiquitous inactivation of *fh*, although in this case, larvae could survive up to 45 days allowing some individuals to become a full 2-fold larger (12). We quantified the level of *fh* mRNAs and observed a reduction of 91% in fh-200GAA male larvae compared to the level in control larvae of the same age (Fig. 1E), confirming that the GAA expansion with adjacent human intronic sequences strongly reduced the level of frataxin transcripts. Then, we aimed to study the impact of the expansion on biochemical features of the disease. Cytosolic and mitochondrial aconitases are ISC enzymes the activity of which has been shown to be reduced in both human patients and several models of the disease, including Drosophila models (13,14). We observed that aconitase activity was decreased by 29% in *fh-200GAA* larvae (Fig. 1F).

fh-200GAA adults obtained at low temperature are short lived and exhibit locomotor defects

When raised at 16°C, fh-200GAA male larvae still presented defective growth, but to a lower extent compared to the 26°C experiments: their weight was only 15.5% lower compared to control larvae of the same age (Fig. 2A). Pupariation was also delayed but 89% of the larvae formed pupae (Fig. 2B) and 16% managed to reach the adult stage. However, the viability of these adult escapers was strongly affected. When flies were kept at 16°C at the adult stage, their median lifespan was of 10 days (Supplementary Material, Table S1). When these flies were transferred at 26°C after emergence, their median lifespan was of only 3 days compared to 51 days for control flies (Fig. 2C). Their locomotor capacity, evaluated by their ability to climb, was also strongly reduced (Fig. 2D). The level of *fh* mRNAs in these fh-200GAA adult males, quantified just after emergence, was 75% lower compared to control adults raised in the same conditions (Fig. 2E). These results show that the phenotypes of fh-200GAA flies are strongly temperature-dependent. This might be due nonexclusively to a lower requirement of frataxin due to slow metabolism or to a lower impact of GAA expansion on *fh* transcriptional level at low temperature. We quantified the *fh* mRNA levels in control and fh-200GAA larvae raised at 20 or 26°C (Supplementary Material, Fig. S1). First, we observed that *fh* expression was lower at 20°C compared to 26°C in control larvae

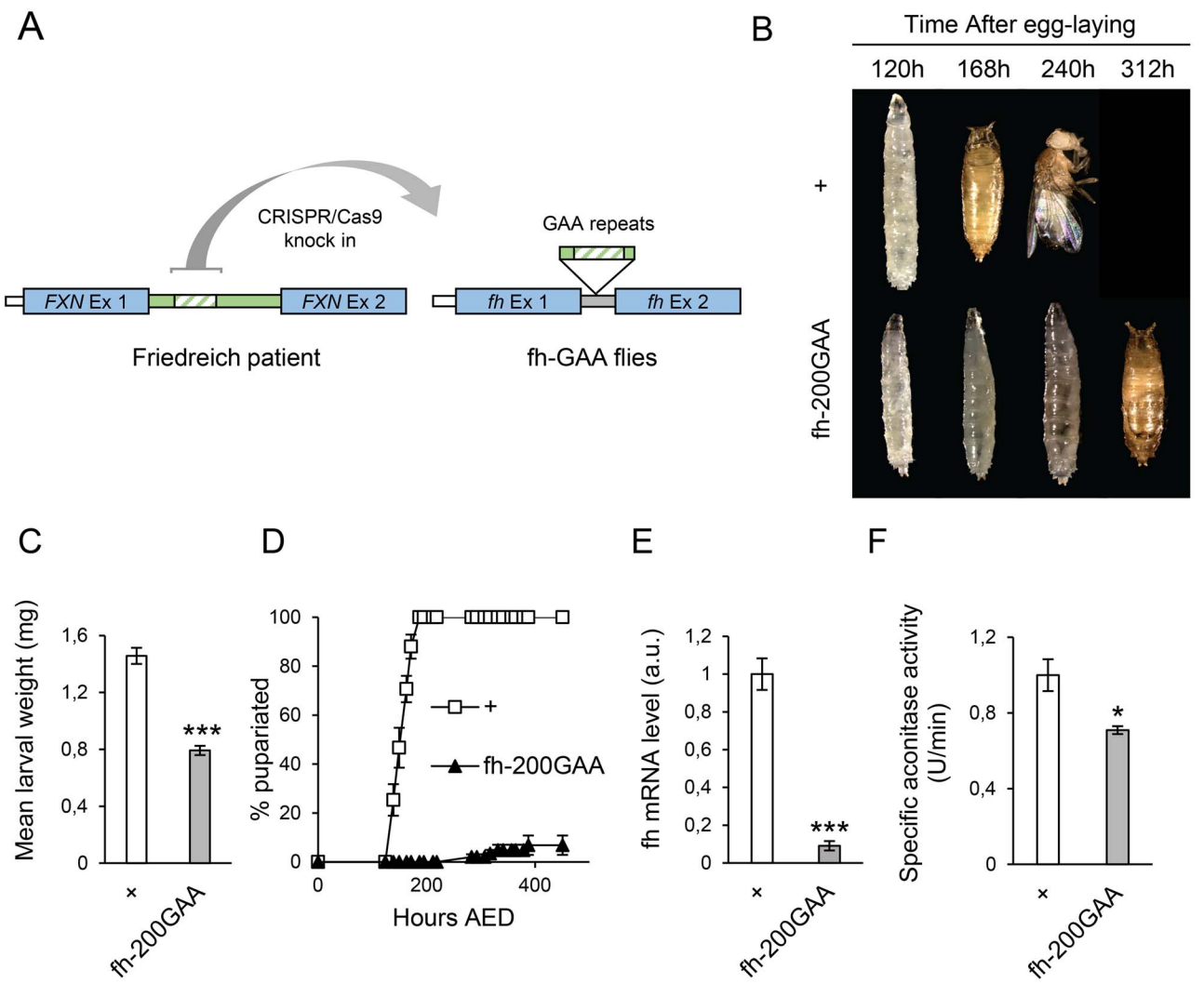

Figure 1. Generation of fh-200GAA flies and developmental phenotypes at 26°C. (**A**) The expansion of around 200 GAA, obtained by PCR on genomic DNA of a FA patient, is inserted in the intron of the Drosophila *fh* gene using the CRISPR/Cas9 technology. (**B**) Pictures of larvae, pupae and adults at different timepoints after egg laying. (**C**) Larval weight 144 h after egg-laying. The mean larval weight ± SEM is calculated on five samples of five control and 10 fh-200GAA male larvae. Significant difference with controls is shown (***$P < 0.001$, unpaired *t*-test). (**D**) Timing of pupariation. Third instar male larvae were transferred in new vials 120 h after egg laying. The percentage of individual reaching the pupal stage over time is indicated. +, $n = 75$; fh-200GAA, $n = 146$. (**E**) Quantification of *fh* transcripts. qRT-PCR were performed on whole RNA extracts from larvae 144 h AED. Significant difference with controls is shown (***$P < 0.001$, unpaired *t*-test). (**F**) Aconitase activity of fh-200GAA and control (+) larvae. Total (mitochondrial and cystolic) aconitase activity was measured on three independent samples of either 120 fh-GAA or 50 control third instar larvae. Mean values are indicated (*$P < 0.05$; unpaired *t*-test). The genotypes are as followed: +, w^{1118}/Y; fh-200GAA, *yw,fh-200GAA/Y*.

and second that the temperature did not statistically modify the *fh* level in fh-200GAA larvae. Altogether, this suggests that the impact of temperature on the phenotype strength is due to a lower need for frataxin when the temperature drops, due to weaker metabolic requirements.

A shorter expansion of 18 GAA induces weaker phenotypes, also temperature dependent

Next, we generated a Drosophila line with only 18 GAA, to determine if the length of the GAA expansion impacts the strength of the phenotypes. At 26°C, when fh-200GAA flies were unable to reach the adult stage, fh-18GAA adult flies were viable, although with a short median lifespan of only 7.4 days (Supplementary Material, Table S1). At 16°C, fh-18GAA flies exhibited a median lifespan of 44 days, whereas fh-200GAA flies lived only 10 days. Thus, the lower number of GAA led to weaker effects on viability and lifespan. Inverse correlation between temperature and median lifespan is occurring naturally in Drosophila flies, so we calculated the ratio between the median lifespans of fh-18GAA and control flies. This ratio was of 0.17 at 26°C, 0.26 at 23°C and 0.28 at 20°C, showing that the longevity phenotype of the fh-18GAA line was temperature dependent. Noticeably, the 18 GAA expansion was already pathological in flies. Accordingly, the *fh* mRNA level was reduced in fh-18GAA larvae, although to a lesser extent compared to fh-200 GAA larvae raised at the same temperature (Supplementary Material, Fig. S2). This suggests a stronger susceptibility in flies to GAA expansions compared to humans, possibly due to the shorter size of the Drosophila intron compared to the human one. Alternatively, we cannot exclude an impact on frataxin expression of the adjacent human intronic sequences also inserted in the *fh* intron.

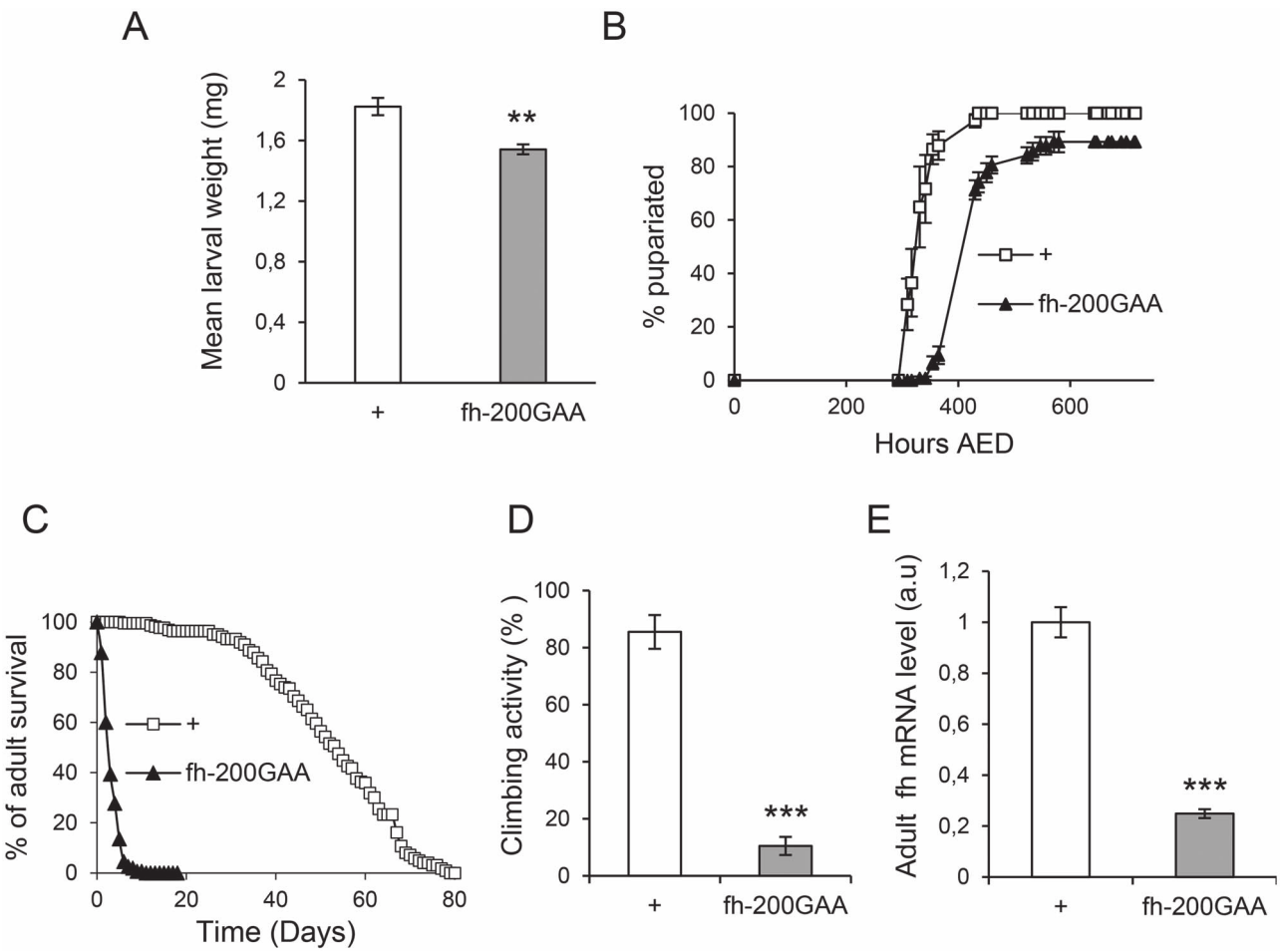

Figure 2. Developmental and adult phenotypes of fh-200GAA flies raised during development at 16°C. (**A**) Mean larval weight. The mean larval weight ± SEM 330 h after egg-laying is calculated on five samples of five larvae for controls and five samples of seven larvae for fh-200GAA. Significant difference is indicated (**$P < 0.01$, unpaired *t*-test). (**B**) Timing of pupariation. Third instar larvae were transferred in new vials 290 h after egg laying. The percentages of individual reaching the pupal stage over time is indicated. +, $n = 74$, fh-200GAA, $n = 139$. (**C**) Survival curve of fh-200GAA adult male escapers. Mean and median lifespan were, respectively, 51 and 53 days for + and 2.6 and 3 days for fh-200GAA flies. +, $n = 223$; fh-200GAA, $n = 155$. (**D**) Climbing activity was assessed by negative geotaxis assays. The ability of 1-day-old male flies to climb up the wall of a tube after being made to fall to the bottom by tapping was evaluated on 4 samples of 10 flies for each genotype. The percentage of flies that climbed more than 3 cm after 10 s is indicated (***$P < 0.001$, unpaired *t*-test). (**E**) Quantification of *fh* transcripts in fh-200GAA adult male escapers. qRT-PCR were performed on whole RNA extracts from 1-day-old male fh-200GAA and control males. Significant difference with controls is shown (***$P < 0.001$, unpaired *t*-test). The genotypes are as followed: +, w^{1118}/Y; fh-200GAA, *yw,fh-200GAA/Y*. Development of fh-200GAA and control flies was performed at 16°C for all experiments and adult escapers transferred at 26°C after emergence.

Developmental and adult phenotypes of fh-GAA flies are rescued by frataxin overexpression

We used an inducible system to overexpress *fh* and determine if it was sufficient to rescue the phenotypes of fh-200GAA flies. In *fh-200GAA/Y; daGS>UAS-fh* male flies, frataxin was expressed ubiquitously under control of the RU486-inducible daGS driver, only if RU486 was provided in the food (Fig. 3A). Without RU486 treatment, these larvae did not reach the adult stage and presented defective and delayed pupariation (Fig. 3B). It should be noted that this developmental phenotype was weaker compared to *fh-200GAA/Y* flies, with 57% of the larvae reaching the pupal stage compared to 7%, probably due to a small tissue-specific developmental leakage of the daGS driver in absence of RU486, which was not detectable by qPCR on whole larval extracts (Supplementary Material, Fig. S3).

When RU486 was added in the food during development, these larvae developed normally and reached the adult stage without any growth or pupariation delay (Fig. 3B). The *fh-200GAA/Y;daGS>UAS-fh* adult males obtained this way were then subdivided in two categories, with sustained RU486 treatment or not. The population of flies fed with RU486 during the adult stage had a lifespan close to a wild-type level, with a mean lifespan of 33 days. The untreated adult population presented a short lifespan with a mean lifespan of only 11.5 days (Fig. 3C). These flies also exhibited a strong locomotor defect in climbing assays, which was not observed in flies with sustained RU486 treatment (Fig. 3D).

Consequently, we conclude that the developmental and adult phenotypes of fh-200GAA flies previously described are indeed caused by the depletion of the frataxin protein. Moreover, the use of the inducible system to overexpress *fh* only during development appears to be highly pertinent to study the effects of partial frataxin depletion specifically during the adult stage.

In order to complete our comparison between the 18 GAA and the 200 GAA expansions, we applied the same rescue system in *fh-18GAA/Y; daGS>UAS-fh* flies. Here again, we observed weaker effects with the 18 GAA compared to the 200 GAA expansion, with a median lifespan of 29 days in these flies treated with

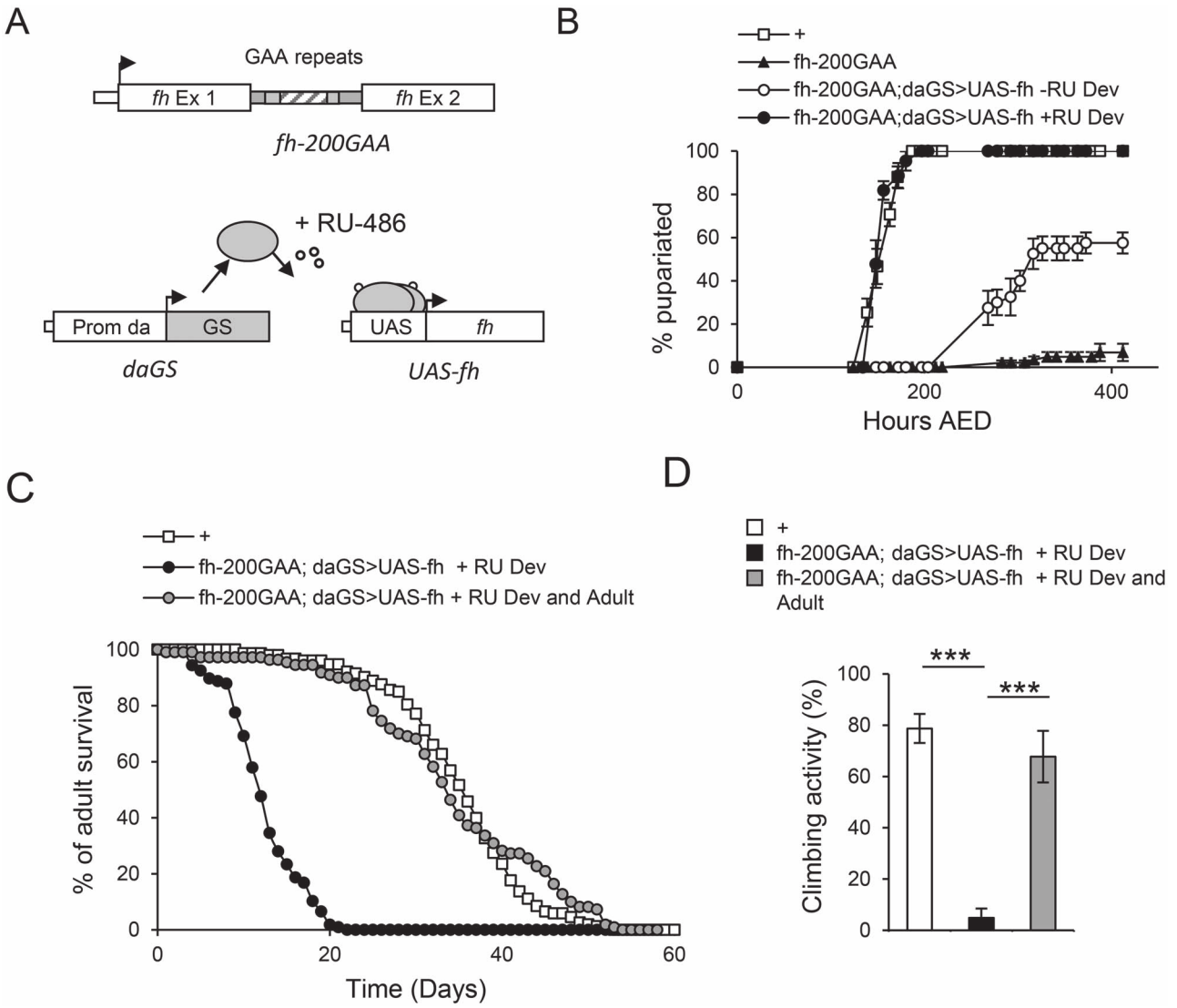

Figure 3. Phenotypic rescue by frataxin ubiquitous overexpression. (**A**) Expression of frataxin under control of the GS inducible system. *daGS* and *UAS-fh* transgenes allow RU-dependent inducible expression of the frataxin protein by the GeneSwitch protein (GS), ubiquitously expressed under control of the daughterless promoter. (**B**) Timing of pupariation. Third instar larvae were transferred in new vials 135 h after egg laying. The percentage of individual reaching the pupal stage over time is indicated. Larvae were raised on medium containing RU (10 µg/ml, +RU Dev) to overexpress frataxin or not (−RU Dev). +, $n = 74$; fh-GAA, $n = 146$; *fh-200GAA;daGS>UAS-fh* −RU Dev, $n = 40$, *fh-200GAA;daGS>UAS-fh* +RU Dev: $n = 44$. (**C**) Survival curves of *fh-200GAA;daGS>UAS-fh* adult males obtained by RU treatment (10 µg/ml) to overexpress frataxin during development. Adults were then treated with RU (100 µg/ml, +RU Dev and adult) to maintain frataxin expression or not (+RU Dev). Median lifespan and number of flies were as followed: +: 34 days, $n = 153$; *fh-200GAA;daGS>UAS-fh* +RU Dev: 11.4 days, $n = 70$; *fh-200GAA;daGS>UAS-fh* +RU Dev and Adult: 33.3 days, $n = 110$. (**D**) Climbing activity of the same flies. The percentage of 8-day-old flies that climbed more than 5 cm after 8 s is indicated (***$P < 0.001$, One-way ANOVA with *post hoc* Tukey multiple comparison test). +: $n = 38$; *fh-200GAA;daGS>UAS-fh* +RU Dev: $n = 44$; *fh-200GAA;daGS>UAS-fh* +RU Dev and Adult: $n = 45$. All the experiments were performed at 26°C (development and adulthood).

RU486 during development (Supplementary Material, Fig. S4A). They did not show significant decrease of climbing activity at 8 days of age (Supplementary Material, Fig. S4B). When the RU486 treatment was maintained at the adult stage, the median lifespan was increased up to 36 days, showing that the reduced longevity of fh-18GAA flies is due to frataxin deficiency as expected (Supplementary Material, Fig. S4A).

Transcriptome analysis of frataxin-deficient flies

In order to obtain a complete picture of gene expression modifications induced by frataxin deficiency, we analyzed the transcriptome of *fh-200GAA/Y; daGS>UAS-fh* male flies treated with RU486 only during development (referenced below as frataxin-deficient flies), and used *daGS* and *daGS>UAS-fh* male flies, treated similarly with RU486, as references. Fly heads were used to enrich the samples in nervous tissue. Quantification of the *fh* transcript level by qRT-PCR showed that the frataxin transcriptional level in frataxin-deficient fly heads was below 40% of control levels at three different time points (4, 8 and 12 days of adulthood) (Fig. 4A). Thus, we used 5-day-old frataxin deficient flies to identify early transcriptional modifications linked to the disease. RNA-Seq analysis identified 230 upregulated and 88 downregulated genes in these frataxin-deficient flies compared to controls (Supplementary Material, Table S2).

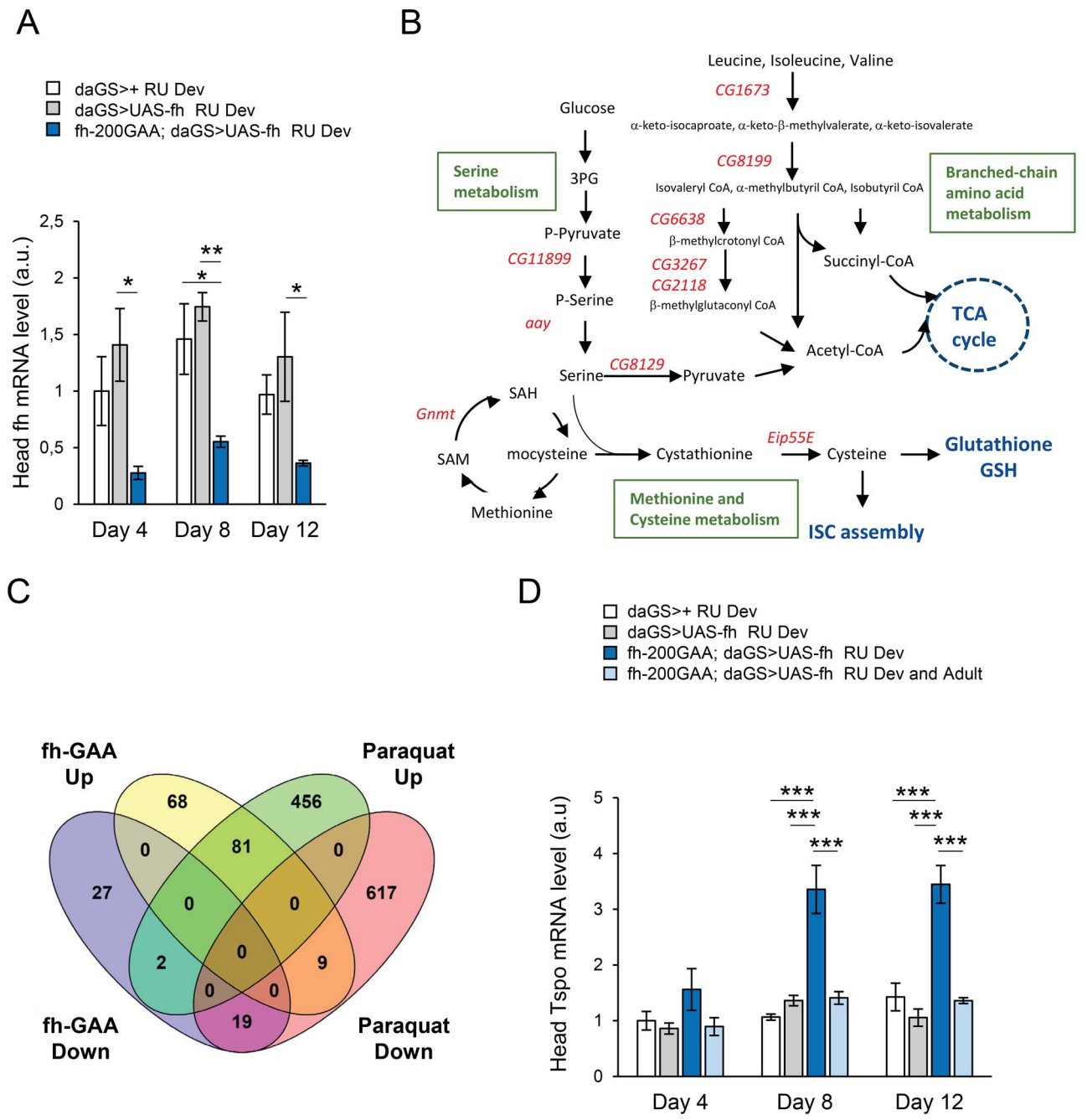

Figure 4. Transcriptome analysis of frataxin-deficient flies. (**A**) Quantification of *fh* transcripts. *fh-200GAA; daGS>UAS-fh* (frataxin-deficient flies), *daGS>+* and *daGS>UAS-fh* male flies (control flies) were treated with RU486 only during development (10 µg/ml in fly food). qRT-PCR were performed on whole RNA extracts of 4-day, 8-day and 12-day-old male adult heads (5 samples of 50 heads per condition). *fh* level is expressed as relative transcript level compared to 4-day-old *daGS>UAS-fh* control flies. Significant difference between *fh-200GAA; daGS>UAS-fh* and controls are shown (*$P < 0.05$, **$P < 0.01$, One-way ANOVA with *post hoc* Tukey multiple comparison test, performed for each age group). (**B**) Schematic diagram showing in red the genes involved in amino-acid metabolism that are upregulated in frataxin-deficient flies. *CG1673* and *CG8199* are genes likely encoding enzymes involved in the first steps of isoleucine, leucine and valine catabolism [BCAT, Branched-chain amino-acid aminotransferase and member of the branched-chain α-keto acid dehydrogenase complex (BCKDC)]. *CG6638*, *CG3267* and *CG2118* likely encode enzymes involved in the following steps of leucine catabolism (IVDC, isovaleryl CoA dehydrogenase; MCCs, methylcrotonoyl-CoA carboxylase 1 and 2 enzymes). (**C**) Venn diagram showing the number of genes deregulated in frataxin-deficient flies and in flies submitted to paraquat-induced oxidative stress (29). (**D**) Quantification of *Tspo* transcripts. All flies were treated with RU486 during development (10 µg/ml in fly food). *fh-200GAA; daGS>UAS-fh* flies were either treated with RU486 during adulthood to maintain frataxin overexpression (RU Dev and Adult) or not (RU Dev). qRT-PCR were performed on whole RNA extracts of 4-day, 8-day and 12-day-old male adult heads (5 samples of 50 heads per condition). Significant differences are shown (***$P < 0.001$, One-way ANOVA with *post hoc* Tukey multiple comparison test performed for each age group). All the flies used for Deep sequencing and qRT-PCR experiments were raised at 26°C during both development and adulthood.

We used the PANTHER platform to identify, in these two sets of genes, over-representation of cellular components, molecular functions or biological processes. In the set of upregulated genes, 42 encoded mitochondrial proteins, showing a strong enrichment in this subcellular compartment (Supplementary Material, Table S3). Analysis of molecular functions revealed

that cofactor binding, catalytic and oxidoreductase activities were the most significantly enriched classes with, respectively, 30, 135 and 49 overexpressed genes. The oxidoreductase category contains 12 genes encoding Cytochrome P450 (CYP) proteins, the two Drosophila ferredoxin genes (*Fdxh* and *Fdx2*), the two flavin-containing Mono-oxygenases (*Fmo-1* and *Fmo-2*), the lactate dehydrogenase *ImpL3*, *Nmdmc* (a gene involved in the purine biosynthetic pathway) and several genes encoding proteins with peroxidase activity, including the peroxidases *PHGPx* and *Jafrac-1*. Interestingly, overexpression of *Nmdmc*, *PHFPx* and *Jafrac-1* were previously shown to be protective against oxidative stress in flies (22–24). Overexpression of the genes encoding ferredoxins might also be interpreted as an attempt to improve ISC assembly, considering that the FDX2 ferredoxin is an essential enzyme in this process in mammals (25). The glutathione-S-transferase (GST) activity was also over-represented. GST is evolutionary-conserved detoxification enzymes essential for cellular protection against oxidative damage. Upon the 39 genes referenced in the PANTHER database for the GST activity class, 11 were overexpressed in frataxin-deficient flies. These genes mainly belong to the Delta and Epsilon GST classes, with, respectively, five (*GstD1, D2, D5, D9*) and four (*GstE1, E7, E8, E10*) overexpressed genes. Delta and Epsilon GSTs are the most numerous GSTs in *Drosophila melanogaster* (26), mainly located on two genomic clusters, suggesting shared mechanisms of transcriptional regulation. The biological process analysis detected an enrichment in the metabolic process category. In agreement with the molecular function analysis, oxidation–reduction and glutathione metabolic processes were over-represented. In addition, enrichment was observed for several biological processes linked to amino-acid metabolism (Fig. 4B). This includes two genes involved in methionine and cysteine metabolism: *Gnmt*, encoding the enzyme that catalyzes the conversion of S-adenosyl-methionine in S-adenosylhomocysteine (27) and *Eip55E*, encoding a putative cystathionine gamma-lyase involved in the biosynthesis of cysteine. Thus, these gene deregulations could be interpreted as an attempt to increase the pool of cysteine, either to increase the production of glutathione that is derived from cysteine or to boost ISC assembly. Strikingly, five genes encoding enzymes involved in a branched amino-acid (BCAA) catabolism, a process ultimately leading to the production of acetyl Co-A or succinyl-CoA, were also overexpressed. Genes involved in serine metabolism were also upregulated, in particular *CG11899* and *aay*, involved in serine biosynthesis, and *CG8129* likely encoding a serine/threonine dehydratase that breaks down serine and threonine to pyruvate. Overall, these sets of upregulated genes strongly suggested that frataxin deficiency induced a transcriptional response to boost oxidative stress defense and Fe–S biosynthesis along with a metabolic state to replenish TCA cycle intermediates.

The set of downregulated genes is more limited. The cellular component enrichment analysis highlighted the extracellular category (Supplementary Material, Table S4). Interestingly, this includes *Tsf1*, encoding transferrin, a secreted protein involved in iron trafficking, and previously shown to be induced upon iron depletion and repressed following iron overloading (28). The molecular function analysis revealed enrichment in heme and iron binding due to down regulation of nine Cytochrome P450. Observing opposite transcriptional responses within the CYP family may seem surprising but has indeed already been observed in flies submitted to oxidative stresses (29,30).

Transcriptomic signatures of frataxin deficiency are mainly related to oxidative stress

We next compared the transcriptome of frataxin-deficient flies with transcriptomes of flies submitted to various stresses. Confirming our previous hypothesis, we observed, in the set of genes deregulated in fh-GAA flies, a significant and strong enrichment of genes deregulated in similar ways following paraquat (29), H_2O_2 (31) and hyperoxia oxidative stresses (30) (Fig. 4C, Table 1 and Supplementary Material, Table S5). For instance, 81 genes upregulated in fh-GAA flies were also upregulated in flies fed with paraquat, and inversely 19 genes down regulated in fh-GAA flies were also under expressed following paraquat treatment (Fig. 4C). An enrichment in the fh-GAA overexpressed genes was also observed in genes also upregulated following tunicamycin treatment, which induces an endoplasmic reticulum (ER) stress (Table 1). Nrf2 and FOXO transcription factors (TFs) are evolutionary conserved major regulators of cellular defenses against oxidative stress (32). To determine if gene deregulation in fh-GAA flies could be in part dependent on these transcription factors, we compared our sets of genes with transcriptomic studies on flies overexpressing *CncC* (the orthologue of *Nrf2*) (33) and on flies mutants for *dfoxo* (34). We did not observe strong and significant enrichments in foxo-dependent genes in both up-and down-regulated genes from fh-GAA flies. In contrast, significant enrichments in genes deregulated by *CncC* overexpression were observed but without a clear directional effect. Notably, the fh-200GAA induced genes were enriched in genes that were both induced and repressed by CncC overexpression. Overall, these data suggest that these two TFs have a limited role in the transcriptional modifications induced by frataxin deficiency in fh-GAA flies. We also made comparison with a transcriptomic study performed on mutants for the estrogen-related receptor (ERR), a transcriptional regulator known to be involved in the regulation of metabolic processes, in particular mitochondrial biogenesis and function (35). Although this previous study was performed on drosophila larvae, which could be a limitation for the comparison with our data, a number of induced and repressed genes in fh-200GAA were deregulated in a similar way in ERR mutant. This suggests that ERR activity might be affected in frataxin-deficient flies.

Tspo expression is progressively induced in frataxin-deficient flies

We reasoned that some of the transcriptional signatures described above may constitute relevant markers of disease progression. We focused particularly on the *Tspo* gene, detected as overexpressed in our DeespSeq experiments. TSPO is an evolutionarily conserved protein located on the outer membrane of mitochondria and involved in a wide range of mitochondrial functions, including cholesterol transport, steroid hormone and heme biosynthesis, apoptosis and cell proliferation (36). Human *TSPO* expression increases in different neurodegenerative diseases and has been proposed as a biomarker of brain injury and neuroinflammation (37). Here, we observed a progressive increase of *Tspo* expression in the heads of *fh-200GAA/Y; daGS>UAS-fh* adult frataxin-deficient flies (RU486 treatment during development only), fully rescued by adult frataxin expression (Fig. 4D). Consequently, *Tspo* expression can be used as a molecular marker of progression of phenotypes induced by frataxin-deficiency in our fly model. Noticeably, *Tspo* was not significantly overexpressed in the heads of *fh-18GAA/Y;*

Table 1. Comparisons with other transcriptomic data

	Hyperoxia	Paraquat	H_2O_2	Tunicamycin	*Nrf2/CncC* overexp.	*dfoxo* mutant	*dERR* mutant
Distribution of reference genes							
Induced	375	539	839	108	705	758	340
Repressed	432	644	789	99	692	1266	232
Alls	11 224	7435	11 224	7377	11 224	13 963	11 224
Distribution of genes induced in fh-200GAA flies							
Expected induced	7	11	15	2	14	12	6
Expected repressed	8	14	14	2	14	21	4
Induced	**68**	**81**	**50**	**21**	**32**	**21**	**24**
Repressed	8	9	16	0	35	23	11
Alls	198	158	198	150	230	230	198
P-value induced	**0.0E+00**	**0.0E+00**	**3.8E−15**	**8.9E−16**	**8.8E−06**	7.5E−03	**2.3E−09**
P-value repressed	3.5E−01	−1.1E−01	2.3E−01	−1.3E−01	**2.8E−07**	2.7E−01	9.5E−04
Distribution of genes repressed in fh-200GAA flies							
Expected induced	3	3	6	1	6	5	3
Expected repressed	3	4	6	1	5	8	2
Induced	2	2	1	1	14	8	1
Repressed	**13**	**19**	**15**	**0**	**10**	**3**	**8**
Alls	80	48	80	48	88	88	88
P-value induced	−5.0E−01	−3.1E−01	−1.5E−02	1.6E−01	**3.7E−04**	4.9E−02	−2.5E−01
P-value repressed	**2.1E−06**	**8.6E−10**	**1.2E−04**	−5.2E−01	1.9E−02	−3.7E−02	**9.0E−05**

All datasets were obtained in *Drosophila melanogaster* flies. Comparison was performed between genes induced or repressed in frataxin-deficient flies and genes induced or repressed in various conditions of stresses, noted here as reference genes [Hyperoxia, (30), Paraquat (29), H_2O_2 (31) and tunicamycin (29)] or genetic contexts [*CncC* overexpression (33), *dfoxo* mutant (34), *dERR* mutant (35)]. The first three lines contain the number of genes induced, repressed or detectable in the published reference genes dataset. From these values we calculated the expected distribution for genes either induced (lines 4, 5) or repressed (lines 11, 12) in fh-200GAA flies, assuming no correlation. These expected values were compared to the real distribution (lines 6–8 and 13–15) obtained by comparison between fh-200GAA and reference genes dataset. The probability for the null hypothesis (no correlation between datasets) between expected and real distributions are given in lines 9–10 and 16–17, respectively. *P*-value lower than the stringent threshold of 5.10^{-4} are indicated in bold. See Supplementary Material for more details.

daGS>UAS-fh flies with RU486 developmental treatment (Supplementary Material, Fig. S4C). We have also overexpressed or inactivated by RNA interference *Tspo* in fh-200GAA flies, but in both cases, it failed to rescue the developmental lethality (Supplementary Material, Fig. S5).

Comparison with mammalian transcriptomes

To identify the most relevant transcriptome changes, we compared our data with sets of transcriptomes obtained on mouse tissues (38,39) or on cells from FA patients (40–45) (Supplementary Material, Table S6). Among the 318 differentially expressed genes identified in our study, 256 have at least one mammalian ortholog, with the total of human and mouse orthologs to Drosophila deregulated genes being 610 and 690, respectively. Interestingly, we found that 144 (56%) of these Drosophila genes have a mammalian ortholog, which exhibit a similar transcriptional variation in at least one transcriptome related to FA. Among these genes, 43 have orthologous observed to be similarly deregulated in at least one human and one mouse transcriptome study, defining a strongly conserved core of genes related to FA (Supplementary Material, Table S6). *TSPO* is one of these conserved genes, and has been found overexpressed in peripheral blood of FA patients (43) and in hearts of an inducible mouse model of FA (38), suggesting that it might be of interest as a molecular marker in mammalian models of FA or even in patients.

Panther analysis of these conserved sets of genes showed enrichments in the same biological processes and pathways as those we identified with the whole Drosophila set, notably oxidation–reduction process, glutathione metabolic process and branched-chain amino acid catabolism (Supplementary Material, Table S6). Altogether the common transcriptional modification of genes involved in such processes in three different species and different experimental conditions related to FA could suggest new lines of investigation for the pathology.

Candidate drug screening identified protective effect of NAC treatment

We tested several categories of drugs for their ability to increase the lifespan of frataxin-deficient flies, including anti-oxidants, anti-inflammatory agents, inhibitors of sphingolipid synthesis, TSPO ligands, and compounds with the potential property to increase frataxin level, for a total number of 30 drugs. None of these drugs, tested at a concentration of 10 μM in the fly food, led to a significant increase of lifespan (Supplementary Material, Table S7). TSPO ligands were tested at an additional concentration of 30 μM, which also failed to improve lifespan. Since all the compounds have only been tested at one concentration, we cannot exclude positive effects at other concentrations. For this reason, we tested higher concentrations of one of these drugs, NAC, motivated by the fact that recent aging studies in Drosophila have used concentrations up to 100 mM (46,47). Treatment with a 200 μg/ml food concentration did not lead to significant lifespan improvement. The 400 and 800 μg/ml treatments led to mean lifespan increases of 13 and 35%, respectively, showing a dose-dependent effect (Fig. 5A). We also treated flies continuously (during development and adulthood) and observed a mean lifespan increase of 47%, suggesting a higher protective effect when flies are treated before the adult stage (Fig. 5B). Locomotor ability, assessed with climbing assays, was also improved by both the adult-specific and continuous NAC treatments at 800 μg/ml (Fig. 5C). In order to go further in the characterization of this protective effect, we studied the impact of NAC treatment on oxidative stress resistance of control and frataxin-deficient flies.

We observed a very strong sensitivity of frataxin-deficient flies to oxidative stress induced either by hyperoxia or paraquat, an inhibitor of mitochondrial complex I (Fig. 5D and E). For instance, when the median survival time under hyperoxia was of 122 h in control flies, it was only of 20 h in frataxin-deficient flies. In control flies, NAC treatment failed to significantly improve resistance to hyperoxia and only modestly increased resistance to paraquat by 5.7%. In frataxin-deficient flies, NAC treatment induced a significant increase of survival with both oxidative stresses: the median and mean survival time were increased of 73 and 53%, respectively, under hyperoxia, and of 23 and 29%, respectively, under paraquat treatment. NAC treatment also significantly improved aconitase activity in fh-200GAA larvae (Fig. 5F). In conclusion, NAC treatment appears to improve a large panel of phenotypes including survival, locomotory function, resistance to oxidative stress and aconitase activity.

Discussion

In this study, we describe the generation and characterization of a new Drosophila model of FA with a GAA expansion inserted in the frataxin homolog *fh* gene. This fh-200GAA model reproduces several features of the FA disease, including decreased level of frataxin expression, reduced lifespan and locomotor dysfunction. The level of frataxin transcripts in fh-200GAA hemizygote males was at 10–25% of its normal level, which is in the range of *FXN* transcript level in cells of FA patients (48). Two non-exclusive mechanisms of transcriptional repression of *FXN* by GAA repeats have been proposed: (i) a defect in the transcription elongation rate (48,49), (ii) epigenetic silencing through DNA methylation and histone modifications (48,50,51). Our DeepSeq analysis did not detect significant decreases in the expression of the two genes closely located on both sides of the *fh* locus (*Bap111* and *CG7065* both located at less than 1 kb of the GAA expansion), suggesting that if epigenetic mechanisms are involved, they are limited to short-range silencing effects. The comparison with the fh-18GAA flies indicates that the strength of the phenotypes depends on the length of the GAA expansion. Interestingly, these flies are already symptomatic, suggesting a stronger sensitivity of flies to GAA expansion compared to mammals. Whether or not the human intronic sequences participate in the phenotype, for instance through DNA methylation should also be determined.

In humans and GAA-based mouse models of FA, expanded GAA repeats exhibit intergenerational instability. Somatic instability also occurs, with tissue-specificity (52–55). Here, the fh-200GAA fly model was generated more than 3 years ago (which corresponds to around 50 generations), but both the phenotypes and the size of the expansion (as detected by PCR on genomic DNA) remained fully stable. High stability of other triplet repeats have been also observed in Drosophila (56), suggesting that it is a general feature of this organism. Thus, if this model is not appropriate to study mechanisms of expansion instability, it is highly reliable to study pathological mechanisms and pharmacological interventions with high reproducibility over time.

Considering the strength of the developmental phenotypes, the inability of fh-200GAA flies to reach the adult stage unless frataxin is ectopically expressed during development, and the shortened lifespan of these adult fh-200GAA flies, it appears that Drosophila flies are highly sensitive to frataxin depletion. In order to bypass the preadult lethality, we had to develop a strategy to express frataxin specifically during development. This approach is indeed particularly interesting in that it allowed to specifically study the effects of frataxin depletion in the adult organism, independently of developmental effects. Moreover, by performing RNA-Seq analysis only 5 days after the beginning of the adult stage, it gave us the opportunity to identify early transcriptomic signatures of frataxin deficiency. As mentioned previously, we observed a strong enrichment in genes also deregulated in various conditions of oxidative stresses (29–31). In particular, the nature of a large number of genes over-expressed in fh-200GAA flies (encoding GST, CYP450 and peroxidases) strongly suggests activation of protective mechanisms to fight against oxidative stress insults. Upregulation of the genes encoding the two Drosophila ferredoxin genes might also be due to a transcriptional regulatory loop in response and in an attempt to mitigate decreased ISC biogenesis. Strikingly, we observed overexpression of five genes encoding enzymes of BCAA catabolism (Fig. 4). These amino-acids play essential roles in brain metabolism. They are involved in glutamate metabolism, in protein synthesis and regulation of food intake and can also be utilized as energy substrates in the tricarboxylic acid cycle (57). Interestingly, reduced levels of BCAAs have been reported in patients with Huntington's disease and recently associated with increased levels of several enzymes involved in their catabolism, suggesting upregulation of anaplerotic pathways to compensate brain energy deficits (58). Here, the causes and functional consequences of enhanced BCAA metabolism induced by frataxin-deficiency are unclear but should be further explored. Interestingly, BCAA catabolism is also one of the enriched pathway in the set of the 144 genes, which exhibit similar transcriptional variation in Drosophila and at least one mammalian transcriptome related to FA.

We also detected a progressive and strong activation of *Tspo* gene expression in frataxin-deficient flies. In mammals, *TSPO* is widely expressed, particularly in steroidogenic tissues such as adipose tissue and adrenal cortex, with a low expression in the central nervous system, restricted mostly to glial cells and some neuronal cell types (36,59). Interestingly, *TSPO* expression increases following brain injury and in several neurodegenerative diseases including amyotrophic lateral sclerosis, Huntington's, Parkinson's and Alzheimer's diseases (60). Several TSPO ligands have been developed and evaluated for their neuroprotective properties (59). For instance, Emapunil, a synthetic TSPO ligand, was recently shown to protect against neurodegeneration in a mouse model of Parkinsonism (61). Here, we have tested four TSPO ligands (Emapunil, Etifoxine, Olesoxime and PK11195) but failed to observe, at the tested concentrations, significant effects on the lifespan of frataxin-deficient flies. Modulation of *Tspo* expression by genetic means also failed to rescue the developmental lethality. Consequently, at this stage, we have not been able to show that TSPO could be a therapeutic target for FA. However, *Tspo* increased expression might be of interest as a biomarker of the progression of the disease. Indeed, radiolabeled TSPO ligands have been developed and used to evaluate neuroinflammation in several contexts of neurodegenerative diseases using positron emission tomography (PET) (62,63). As mentioned previously, overexpression of *TSPO* has also been observed in two other transcriptomic studies, including one performed on peripheral blood of FA patients (43), suggesting that *TSPO* level of expression could potentially be used as a blood marker of the progression of the disease.

We performed a candidate drug screening, using the adult fly survival as a readout. For practical reasons (duration of the experiments, number of individuals to be followed in longevity, solubility of the compounds and tolerance of flies to the DMSO solvent), we have tested most of the candidate drugs at one single concentration (10 μM). This could be a limitation of our

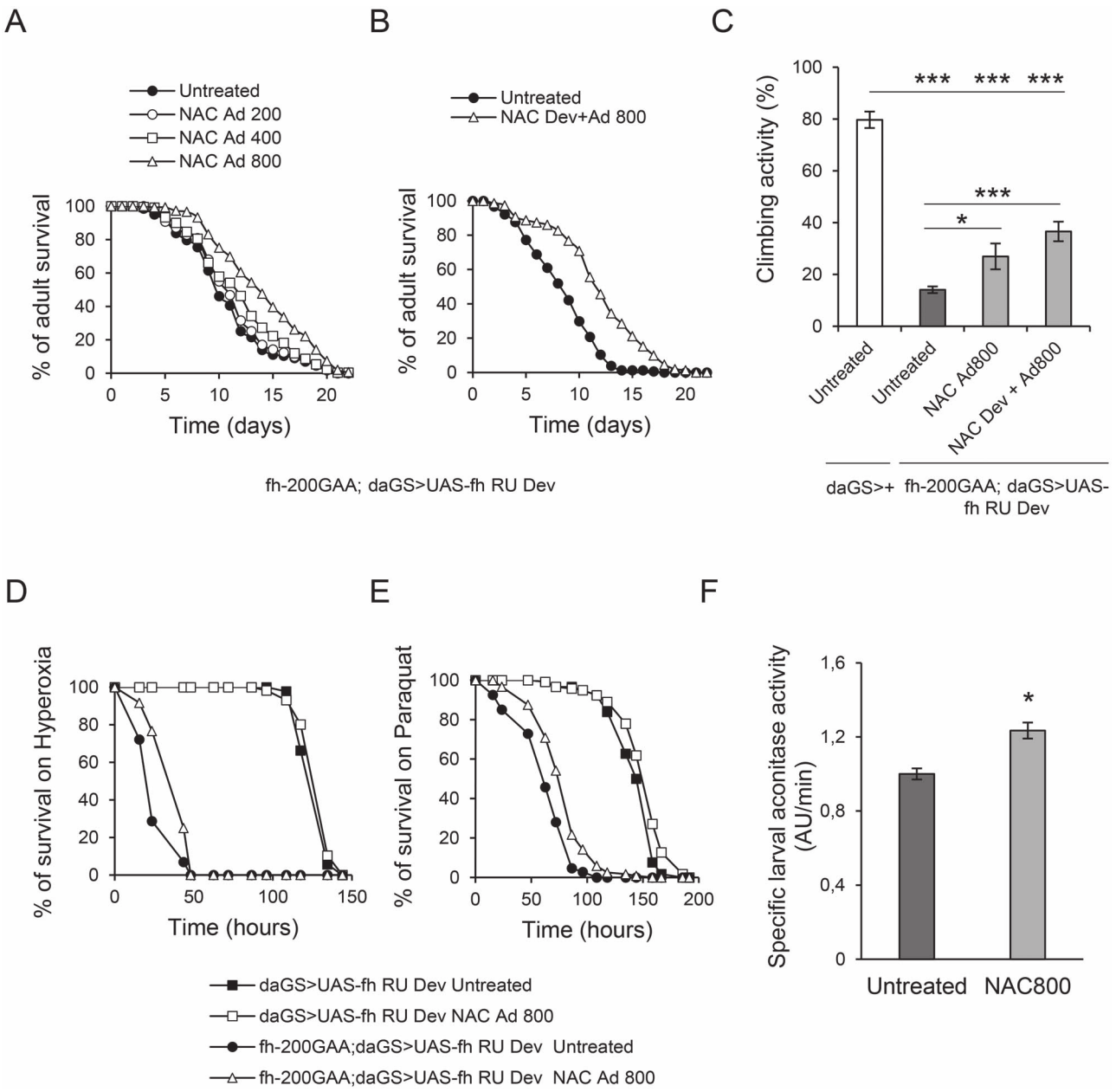

Figure 5. Protective effect of NAC treatment. (**A**) Survival curves of *fh-200GAA;daGS>UAS-fh* adult flies untreated ($n = 143$) or treated with NAC during adulthood at a final concentration in the fly food of 200 µg/ml (NAC Ad 200, $n = 282$), 400 µg/ml (NAC Ad 400, $n = 242$) or 800 µg/ml (NAC Ad 800, $n = 149$). The percentages of mean lifespan increase compared to untreated flies and the *P*-values evaluated by logrank tests are as followed: NAC Ad 200: 5.8%, $P = 2.5{\cdot}10^{-1}$, NAC Ad 400: 12.8%, $P = 1.1{\cdot}10^{-2}$, NAC Ad800: 35.2%, $P = 1.1{\cdot}10^{-9}$. (**B**) Survival curves of *fh-GAA;daGS>UAS-fh* adult flies untreated ($n = 154$) or treated with NAC during adulthood and development at 800 µg/ml (NAC Dev + Ad 800, $n = 151$). NAC treatment increased the mean lifespan of frataxin-deficient flies of 46.9% ($P = 5.15{\cdot}10^{-17}$, logrank test). (**C**) Climbing activity of *daGS>+* (+, $n = 240$) and *fh-200GAA;daGS>UAS-fh* adult flies untreated ($n = 240$) or treated with NAC 800 µg/ml only during adulthood (NAC Ad800, $n = 90$) or during development and adulthood (NAC Dev800 Ad800, $n = 80$). The percentage of flies that climbed more than 3 cm after 10 s is indicated (***$P < 0.001$, *$P < 0.05$, One-way ANOVA with *post hoc* Tukey multiple comparison test). All the flies were treated with RU (10 µg/ml) during development. (**D**) Resistance to hyperoxia of *fh-200GAA;daGS>UAS-fh* adult flies untreated ($n = 115$) or treated with 800 µg/ml of NAC during adulthood ($n = 120$), and of *daGS>UAS-fh* control flies untreated ($n = 89$) or similarly treated with NAC ($n = 116$). Flies were placed under hyperoxia 3 days after emergence. The percentages of mean lifespan increase conferred by NAC treatment for flies of the same genotype and the *P*-values evaluated by logrank tests are as followed: *fh-200GAA;daGS>UAS-fh*: 53.2%, $P = 1.1{\cdot}10^{-9}$, *daGS>UAS-fh*: 1.4%, $P = 9.6{\cdot}10^{-2}$. (**E**) Resistance to paraquat of *fh-200GAA;daGS>UAS-fh* adult flies untreated ($n = 107$) or treated with 800 µg/ml of NAC during adulthood ($n = 120$), and of *daGS>UAS-fh* control flies untreated ($n = 118$) or similarly treated with NAC ($n = 118$). Flies were placed in vials containing 2.5 mM paraquat 3 days after emergence. The percentages of mean lifespan increase conferred by NAC treatment for flies of the same genotype and the *P*-values evaluated by logrank tests are as followed: *fh-200GAA;daGS>UAS-fh*: 29.2%, $P = 4.7{\bullet}10^{-7}$, *daGS>UAS-fh*: 5.7%, $P = 7.0{\bullet}10^{-5}$. (**F**). Aconitase activity of fh-200GA larvae, treated or not with 800 µg/ml of NAC. Total (mitochondrial and cystolic) aconitase activity was measured on three independent samples of 120 fh-GAA or 50 control third instar larvae. Mean values are indicated (*$P < 0.05$, unpaired *t*-test). All flies were raised at 26°C during both development and adulthood.

approach and we do not exclude that some of the drugs might have positive effects with other dosages. Indeed, we identified protective effects of *N*-acetyl-cysteine when provided at higher concentrations. NAC is an old drug with several established clinical applications (paracetamol overdose, mucolytic effect) and is currently widely used as an antioxidant in clinical trials for various applications as diverse as blood, gastrointestinal, cardiovascular, pulmonary, renal or psychiatric diseases (64). The antioxidant properties of NAC are thought to occur by several mechanisms: (i) direct antioxidant effect, as a thiol compound. However, due to low-reaction rate constants, this effect is most likely negligible compared with that of antioxidant enzymes (65), (ii) indirect antioxidant effect by its ability to act as a source of cysteine for increasing the intracellular pool of glutathione (GSH), particularly in contexts of GSH depletion (64), (iii) indirect antioxidant effect by triggering intracellular H_2S and sulfane sulfur production (66). Noticeably, we only observed weak resistance to oxidative stress conferred by NAC treatment in control flies, in agreement with previous studies in Drosophila showing limited *in vivo* antioxidant effects of this compound (46,67). In contrast, we observed that NAC increases the activity of aconitase, an ISC enzyme. NAC could operate indirectly as an antioxidant by preventing destruction of ISC. In the context of frataxin deficiency, NAC could also act directly on ISC assembly, either by providing a source of cysteine to the machinery, or as a sulfide donor. The former hypothesis would be consistent with the transcriptomic analysis attesting the upregulation of two genes encoding enzymes of the cysteine biosynthetic pathway (*Gnmt* and *Eip55E*). If the beneficial effect of NAC treatment involves such specific effects on ISC biosynthesis, it would be highly interesting in the context of the FA disease, since we would have a drug acting on the very upstream events of the pathophysiological cascade.

For now, there is only limited data in the literature on NAC effect in cellular contexts of frataxin deficiency. NAC was shown to enhance viability of frataxin-deficient cells non-committed to the neuronal lineage during retinoic acid-induced neurogenesis (68). NAC amide, a NAC derivative developed to improve lipophilicity and brain penetration, improved defective microtubule stability in an *in vitro* neuronal model of FA (69). NAC has also been reported to be taken by some FA patients for its antioxidant properties more than 10 years ago (70). However, to our knowledge, controlled clinical trials that explore the efficacy of NAC in FA patients have not yet been performed. This study provides for the first time evidences of *in vivo* protective effects, which should stimulate the clinical evaluation of this compound with high therapeutic potential.

Disorders due to expansions of trinucleotide repeats (TNR) account for more than 20 inherited neurological diseases. The expansions could be either in the non-coding part of the affected gene like in FA or in myotonic dystrophy type 1 (DM1), or in the coding region of the gene, like in polyglutamine diseases (Huntington disease, spinocerebellar ataxias) due to CAG expansions. The Drosophila models developed to study dominant TNR diseases are mainly based on expression of mutated human proteins with the UAS/GAL4 system, leading to high expression far from the level of expression of the corresponding endogenous protein. Here, we have shown that the CRISPR/Cas9 technology allows the insertion of TNR directly in the sequence of the gene orthologous to the human affected gene. We believe that our fh-GAA model will be the first of a new generation of endogenous Drosophila models of TNR diseases with improved physiological relevance to study pathophysiological mechanisms and to identify promising therapeutic strategies.

Materials and Methods

Drosophila lines and culture methods

To generate the fh-GAA line, GAA repeats flanked by intronic sequences of *FXN* were amplified by PCR on the genomic DNA NA16216 obtained from Coriell Institute (Camden, NJ, USA) using the following primers: 5′-AATGGATTTCCTGGCAGGACGC-3′ and 5′-GCATTGGGCGATCTTGGCTTAA-3′. This PCR product was inserted in a PUC19 plasmid downstream a 991 pb sequence and upstream a 995 pb of the *fh* locus constituting the homology arms of the donor plasmid, which allowed insertion of the human sequence between positions 13 and 37 of the *fh* intron. The donor plasmid for the fh-18GAA construct was obtained by GAA contraction in NEB5-α *E. coli* (Biolabs). The pCFD4-U6:1_U6:3tandemgRNAs, a gift from Simon Bullock (Addgene plasmid # 49411; http://n2t.net/addgene:49411; RRID:Addgene_49411), was used to express a gRNA with the following protospacer sequence: gGAAACCCCGC-GAGGATTAGC under control of the U6:1 promoter (71). The donor and gRNA plasmids were injected in *yw; attP40(nos-Cas9)/CyO* line by BestGene Inc (Chino Hills, CA, USA). *UAS-fh (w[1]; P{w[+mC] = UAS-fh.A}1)* and the *FM7i* and *FM7tb* lines used to balance the *fh-GAA* X chromosome (*AMPKalpha[3]/FM7i* and *FM7a, P{Tb1}FM7-A*) were obtained from the Bloomington Stock Center. The *daGS* line is described in (72). The fly food used for all experiments contained 82.5 mg/ml yeast, 34 mg/ml corn meal, 50 mg/ml sucrose, 11.5 mg/ml agar, and 27.8 µl/ml methyl 4-hydroxybenzoate (stock solution 200 g/l in ethanol). When required, RU486 was incorporated in the fly food from a 20 mg/ml stock solution in ethanol.

RNA-Seq and differential gene expression analysis

Total RNA was extracted following the protocol described in (73) from heads of 5-day-old male flies, treated with RU486 (10 µg/ml) only during development. Three biological replicates for each genotype were sent to IntegraGen (Genopole, Evry, France) for RNA-Seq analysis. The cDNA synthesis was conducted using the NEBNext® Ultra™ II Directional RNA Library Prep Kit for Illumina®. The libraries were sequenced using a 2x75 bases paired end protocol on Illumina HiSeq4000 instrument. Base calling was performed by using the Real-Time Analysis software sequence pipeline (2.7.7) with default parameters. Quality of reads was assessed for each sample using FastQC (V.0.11.4; http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Mapping to Drosophila genome (BDGP6) was performed by STAR (V.2.5; https://github.com/alexdobin/STAR). The numbers of reads, which map to each gene were calculated by using 'HTSeq count' and used to figure out the fragments per kilobase million (FPKM) for each gene. Analysis of differentially expressed genes was performed using the R package DESeq2. Enrichments of gene ontology (GO) terms from the 'Cellular Component', 'Molecular Function' and 'Biological Process' classes, were calculated using the PANTHER tool (version 14.1). See Supporting information for the comparisons with other published transcriptomes.

Quantification of transcripts by qRT-PCR

RNA extractions were performed with the PicoPureTM RNA Isolation Kit (Thermo Fisher Scientific) and treated with dsDNase (Thermo Fisher Scientific) according to the manufacturer's instructions. cDNAs were synthetized from isolated total RNA samples using SusperScript™ III Reverse Transcriptase (Thermo

Fisher Scientific). qPCRs were performed with the qPCR Mix (Promega) on a LightCycler480 (Roche). The ribosomal gene *rp49* was used as an internal reference for normalization. The primers used for amplifications were for the *fh* gene: 5′-ACACCCTGGACGCACTGT-3′ and 5′-CCAGGTTCACGGTTAGCAC-3′, for the *Tspo* gene: 5′-TCGGGCAGCACAACATCAAG-3′ and 5′-GTACGAAGAGCAATCCAGCG-3′, and for the *rp49* gene: 5′-CCGCTTCAAGGGACAGTATCT-3′ and 5′-CACGTTGTGCACCAGGA-ACTT-3′. Quantifications were made on three to five independent biological samples with five technical replicates for each biological sample.

Lifespan experiments and NAC treatment

Male flies were collected within 24 h of eclosion under brief CO_2 anesthesia, housed in groups of 30, and raised at 26°C under a 12–12 h light–dark cycle. They were transferred every 2 days on fresh food, and dead flies were counted. For NAC treatment, the drug (Sigma Aldrich) was incorporated in the fly food at 37°C from a stock solution (80 mg/ml in water) prepared extemporaneously.

Supplementary Material

Supplementary Material is available at *HMG* online.

Conflict of Interest Statement. None declared.

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References

1. Santos, R., Lefevre, S., Sliwa, D., Seguin, A., Camadro, J.M. and Lesuisse, E. (2010) Friedreich ataxia: molecular mechanisms, redox considerations, and therapeutic opportunities. *Antioxid. Redox Signal.*, **13**, 651–690.
2. Durr, A., Cossee, M., Agid, Y., Campuzano, V., Mignard, C., Penet, C., Mandel, J.L., Brice, A. and Koenig, M. (1996) Clinical and genetic abnormalities in patients with Friedreich's ataxia. *N. Engl. J. Med.*, **335**, 1169–1175.
3. Harding, A.E. (1981) Friedreich's ataxia: a clinical and genetic study of 90 families with an analysis of early diagnostic criteria and intrafamilial clustering of clinical features. *Brain*, **104**, 589–620.
4. Pandolfo, M. (2009) Friedreich ataxia: the clinical picture. *J. Neurol.*, **256**, 3–8.
5. Tsou, A.Y., Paulsen, E.K., Lagedrost, S.J., Perlman, S.L., Mathews, K.D., Wilmot, G.R., Ravina, B., Koeppen, A.H. and Lynch, D.R. (2011) Mortality in Friedreich ataxia. *J. Neurol. Sci.*, **307**, 46–49.
6. Weidemann, F., Rummey, C., Bijnens, B., Stork, S., Jasaityte, R., Dhooge, J., Baltabaeva, A., Sutherland, G., Schulz, J.B. and Meier, T. (2012) The heart in Friedreich ataxia: definition of cardiomyopathy, disease severity, and correlation with neurological symptoms. *Circulation*, **125**, 1626–1634.
7. Braymer, J.J. and Lill, R. (2017) Iron-sulfur cluster biogenesis and trafficking in mitochondria. *J. Biol. Chem.*, **292**, 12754–12763.
8. Llorens, J.V., Soriano, S., Calap-Quintana, P., Gonzalez-Cabo, P. and Molto, M.D. (2019) The role of iron in Friedreich's ataxia: insights from studies in human tissues and cellular and animal models. *Front. Neurosci.*, **13**, 75.
9. Tamarit, J., Obis, E. and Ros, J. (2016) Oxidative stress and altered lipid metabolism in Friedreich ataxia. *Free Radic. Biol. Med.*, **100**, 138–146.
10. Aranca, T.V., Jones, T.M., Shaw, J.D., Staffetti, J.S., Ashizawa, T., Kuo, S.H., Fogel, B.L., Wilmot, G.R., Perlman, S.L., Onyike, C.U. *et al.* (2016) Emerging therapies in Friedreich's ataxia. *Neurodegener. Dis. Manag.*, **6**, 49–65.
11. Ugur, B., Chen, K. and Bellen, H.J. (2016) Drosophila tools and assays for the study of human diseases. *Dis. Model. Mech.*, **9**, 235–244.
12. Anderson, P.R., Kirby, K., Hilliker, A.J. and Phillips, J.P. (2005) RNAi-mediated suppression of the mitochondrial iron chaperone, frataxin, in Drosophila. *Hum. Mol. Genet.*, **14**, 3397–3405.
13. Edenharter, O., Schneuwly, S. and Navarro, J.A. (2018) Mitofusin-dependent ER stress triggers glial dysfunction and nervous system degeneration in a Drosophila model of Friedreich's ataxia. *Front. Mol. Neurosci.*, **11**, 38.
14. Llorens, J.V., Navarro, J.A., Martinez-Sebastian, M.J., Baylies, M.K., Schneuwly, S., Botella, J.A. and Molto, M.D. (2007) Causative role of oxidative stress in a Drosophila model of Friedreich ataxia. *FASEB J.*, **21**, 333–344.
15. Navarro, J.A., Ohmann, E., Sanchez, D., Botella, J.A., Liebisch, G., Molto, M.D., Ganfornina, M.D., Schmitz, G. and Schneuwly, S. (2010) Altered lipid metabolism in a Drosophila model of Friedreich's ataxia. *Hum. Mol. Genet.*, **19**, 2828–2840.
16. Palandri, A., L'Hote, D., Cohen-Tannoudji, J., Tricoire, H. and Monnier, V. (2015) Frataxin inactivation leads to steroid deficiency in flies and human ovarian cells. *Hum. Mol. Genet.*, **24**, 2615–2626.
17. Palandri, A., Martin, E., Russi, M., Rera, M., Tricoire, H. and Monnier, V. (2018) Identification of cardioprotective drugs by medium-scale in vivo pharmacological screening on a Drosophila cardiac model of Friedreich's ataxia. *Dis. Model. Mech.*, **11**.
18. Tricoire, H., Palandri, A., Bourdais, A., Camadro, J.M. and Monnier, V. (2014) Methylene blue rescues heart defects in a Drosophila model of Friedreich's ataxia. *Hum. Mol. Genet.*, **23**, 968–979.
19. Chen, K., Lin, G., Haelterman, N.A., Ho, T.S., Li, T., Li, Z., Duraine, L., Graham, B.H., Jaiswal, M., Yamamoto, S. *et al.* (2016) Loss of Frataxin induces iron toxicity, sphingolipid synthesis, and Pdk1/Mef2 activation, leading to neurodegeneration. *eLife*, **5**.
20. Monnier, V., Llorens, J.V. and Navarro, J.A. (2018) Impact of Drosophila models in the study and treatment of Friedreich's ataxia. *Int. J. Mol. Sci.*, **19**.
21. Dietzl, G., Chen, D., Schnorrer, F., Su, K.C., Barinova, Y., Fellner, M., Gasser, B., Kinsey, K., Oppel, S., Scheiblauer, S. *et al.* (2007) A genome-wide transgenic RNAi library for conditional gene inactivation in Drosophila. *Nature*, **448**, 151–156.
22. Yu, S., Jang, Y., Paik, D., Lee, E. and Park, J.J. (2015) Nmdmc overexpression extends Drosophila lifespan and reduces levels of mitochondrial reactive oxygen species. *Biochem. Biophys. Res. Commun.*, **465**, 845–850.
23. Missirlis, F., Rahlfs, S., Dimopoulos, N., Bauer, H., Becker, K., Hilliker, A., Phillips, J.P. and Jackle, H. (2003) A putative glutathione peroxidase of Drosophila encodes a thioredoxin peroxidase that provides resistance against oxidative stress but fails to complement a lack of catalase activity. *Biol. Chem.*, **384**, 463–472.

24. Lee, K.S., Iijima-Ando, K., Iijima, K., Lee, W.J., Lee, J.H., Yu, K. and Lee, D.S. (2009) JNK/FOXO-mediated neuronal expression of fly homologue of peroxiredoxin II reduces oxidative stress and extends life span. *J. Biol. Chem.*, **284**, 29454–29461.
25. Gervason, S., Larkem, D., Mansour, A.B., Botzanowski, T., Muller, C.S., Pecqueur, L., Le Pavec, G., Delaunay-Moisan, A., Brun, O., Agramunt, J. *et al.* (2019) Physiologically relevant reconstitution of iron-sulfur cluster biosynthesis uncovers persulfide-processing functions of ferredoxin-2 and frataxin. *Nat. Commun.*, **10**, 3566.
26. Saisawang, C., Wongsantichon, J. and Ketterman, A.J. (2012) A preliminary characterization of the cytosolic glutathione transferase proteome from *Drosophila melanogaster*. *Biochem. J.*, **442**, 181–190.
27. Obata, F. and Miura, M. (2015) Enhancing S-adenosylmethionine catabolism extends Drosophila lifespan. *Nat. Commun.*, **6**, 8332.
28. Xiao, G., Liu, Z.H., Zhao, M., Wang, H.L. and Zhou, B. (2019) Transferrin 1 functions in iron trafficking and genetically interacts with ferritin in *Drosophila melanogaster*. *Cell Rep.*, **26**, 748–758 e745.
29. Girardot, F., Monnier, V. and Tricoire, H. (2004) Genome wide analysis of common and specific stress responses in adult *Drosophila melanogaster*. *BMC Genomics*, **5**, 74.
30. Landis, G.N., Abdueva, D., Skvortsov, D., Yang, J., Rabin, B.E., Carrick, J., Tavare, S. and Tower, J. (2004) Similar gene expression patterns characterize aging and oxidative stress in *Drosophila melanogaster*. *Proc. Natl. Acad. Sci. USA.*, **101**, 7663–7668.
31. Landis, G., Shen, J. and Tower, J. (2012) Gene expression changes in response to aging compared to heat stress, oxidative stress and ionizing radiation in *Drosophila melanogaster*. *Aging (Albany NY)*, **4**, 768–789.
32. Fuse, Y. and Kobayashi, M. (2017) Conservation of the Keap1-Nrf2 system: an evolutionary journey through stressful space and time. *Molecules*, **22**.
33. Misra, J.R., Horner, M.A., Lam, G. and Thummel, C.S. (2011) Transcriptional regulation of xenobiotic detoxification in Drosophila. *Genes Dev.*, **25**, 1796–1806.
34. Alic, N., Andrews, T.D., Giannakou, M.E., Papatheodorou, I., Slack, C., Hoddinott, M.P., Cocheme, H.M., Schuster, E.F., Thornton, J.M. and Partridge, L. (2011) Genome-wide dFOXO targets and topology of the transcriptomic response to stress and insulin signalling. *Mol. Syst. Biol.*, **7**, 502.
35. Tennessen, J.M., Baker, K.D., Lam, G., Evans, J. and Thummel, C.S. (2011) The Drosophila estrogen-related receptor directs a metabolic switch that supports developmental growth. *Cell Metab.*, **13**, 139–148.
36. Bonsack, F. and Sukumari-Ramesh, S. (2018) TSPO: an evolutionarily conserved protein with elusive functions. *Int. J. Mol. Sci.*, **19**, 1694.
37. Guilarte, T.R. (2019) TSPO in diverse CNS pathologies and psychiatric disease: a critical review and a way forward. *Pharmacol. Ther.*, **194**, 44–58.
38. Chandran, V., Gao, K., Swarup, V., Versano, R., Dong, H., Jordan, M.C. and Geschwind, D.H. (2017) Inducible and reversible phenotypes in a novel mouse model of Friedreich's ataxia. *eLife*, **6**, e30054.
39. Coppola, G., Marmolino, D., Lu, D., Wang, Q., Cnop, M., Rai, M., Acquaviva, F., Cocozza, S., Pandolfo, M. and Geschwind, D.H. (2009) Functional genomic analysis of frataxin deficiency reveals tissue-specific alterations and identifies the PPARgamma pathway as a therapeutic target in Friedreich's ataxia. *Hum. Mol. Genet.*, **18**, 2452–2461.
40. Coppola, G., Burnett, R., Perlman, S., Versano, R., Gao, F., Plasterer, H., Rai, M., Sacca, F., Filla, A., Lynch, D.R. *et al.* (2011) A gene expression phenotype in lymphocytes from Friedreich ataxia patients. *Ann. Neurol.*, **70**, 790–804.
41. Haugen, A.C., Di Prospero, N.A., Parker, J.S., Fannin, R.D., Chou, J., Meyer, J.N., Halweg, C., Collins, J.B., Durr, A., Fischbeck, K. *et al.* (2010) Altered gene expression and DNA damage in peripheral blood cells from Friedreich's ataxia patients: cellular model of pathology. *PLoS Genet.*, **6**, e1000812.
42. Lu, C., Schoenfeld, R., Shan, Y., Tsai, H.J., Hammock, B. and Cortopassi, G. (2009) Frataxin deficiency induces Schwann cell inflammation and death. *Biochim. Biophys. Acta*, **1792**, 1052–1061.
43. Nachun, D., Gao, F., Isaacs, C., Strawser, C., Yang, Z., Dokuru, D., Van Berlo, V., Sears, R., Farmer, J., Perlman, S. *et al.* (2018) Peripheral blood gene expression reveals an inflammatory transcriptomic signature in Friedreich's ataxia patients. *Hum. Mol. Genet.*, **27**, 2965–2977.
44. Napierala, J.S., Li, Y., Lu, Y., Lin, K., Hauser, L.A., Lynch, D.R. and Napierala, M. (2017) Comprehensive analysis of gene expression patterns in Friedreich's ataxia fibroblasts by RNA sequencing reveals altered levels of protein synthesis factors and solute carriers. *Dis. Model. Mech.*, **10**, 1353–1369.
45. Sanchez, N., Chapdelaine, P., Rousseau, J., Raymond, F., Corbeil, J. and Tremblay, J.P. (2016) Characterization of frataxin gene network in Friedreich's ataxia fibroblasts using the RNA-Seq technique. *Mitochondrion*, **30**, 59–66.
46. Shaposhnikov, M.V., Zemskaya, N.V., Koval, L.A., Schegoleva, E.V., Zhavoronkov, A. and Moskalev, A.A. (2018) Effects of N-acetyl-L-cysteine on lifespan, locomotor activity and stress-resistance of 3 Drosophila species with different lifespans. *Aging (Albany NY)*, **10**, 2428–2458.
47. Niraula, P. and Kim, M.S. (2019) N-acetylcysteine extends lifespan of Drosophila via modulating ROS scavenger gene expression. *Biogerontology*, **20**, 533–543.
48. Li, Y., Lu, Y., Polak, U., Lin, K., Shen, J., Farmer, J., Seyer, L., Bhalla, A.D., Rozwadowska, N., Lynch, D.R. *et al.* (2015) Expanded GAA repeats impede transcription elongation through the FXN gene and induce transcriptional silencing that is restricted to the FXN locus. *Hum. Mol. Genet.*, **24**, 6932–6943.
49. Wells, R.D. (2008) DNA triplexes and Friedreich ataxia. *FASEB J.*, **22**, 1625–1634.
50. Sandi, C., Sandi, M., Anjomani Virmouni, S., Al-Mahdawi, S. and Pook, M.A. (2014) Epigenetic-based therapies for Friedreich ataxia. *Front. Genet.*, **5**, 165.
51. Saveliev, A., Everett, C., Sharpe, T., Webster, Z. and Festenstein, R. (2003) DNA triplet repeats mediate heterochromatin-protein-1-sensitive variegated gene silencing. *Nature*, **422**, 909–913.
52. De Michele, G., Cavalcanti, F., Criscuolo, C., Pianese, L., Monticelli, A., Filla, A. and Cocozza, S. (1998) Parental gender, age at birth and expansion length influence GAA repeat intergenerational instability in the X25 gene: pedigree studies and analysis of sperm from patients with Friedreich's ataxia. *Hum. Mol. Genet.*, **7**, 1901–1906.
53. Clark, R.M., De Biase, I., Malykhina, A.P., Al-Mahdawi, S., Pook, M. and Bidichandani, S.I. (2007) The GAA triplet-repeat is unstable in the context of the human FXN locus and displays age-dependent expansions in cerebellum and DRG in a transgenic mouse model. *Hum. Genet.*, **120**, 633–640.
54. De Biase, I., Rasmussen, A., Monticelli, A., Al-Mahdawi, S., Pook, M., Cocozza, S. and Bidichandani, S.I. (2007) Somatic

instability of the expanded GAA triplet-repeat sequence in Friedreich ataxia progresses throughout life. *Genomics*, **90**, 1–5.
55. Anjomani Virmouni, S., Ezzatizadeh, V., Sandi, C., Sandi, M., Al-Mahdawi, S., Chutake, Y. and Pook, M.A. (2015) A novel GAA-repeat-expansion-based mouse model of Friedreich's ataxia. *Dis. Model. Mech.*, **8**, 225–235.
56. Jackson, S.M., Whitworth, A.J., Greene, J.C., Libby, R.T., Baccam, S.L., Pallanck, L.J. and La Spada, A.R. (2005) A SCA7 CAG/CTG repeat expansion is stable in *Drosophila melanogaster* despite modulation of genomic context and gene dosage. *Gene*, **347**, 35–41.
57. Sperringer, J.E., Addington, A. and Hutson, S.M. (2017) Branched-chain amino acids and brain metabolism. *Neurochem. Res.*, **42**, 1697–1709.
58. Andersen, J.V., Skotte, N.H., Aldana, B.I., Norremolle, A. and Waagepetersen, H.S. (2019) Enhanced cerebral branched-chain amino acid metabolism in R6/2 mouse model of Huntington's disease. *Cell. Mol. Life Sci.*, **76**, 2449–2461.
59. Da Pozzo, E., Giacomelli, C., Barresi, E., Costa, B., Taliani, S., Passetti Fda, S. and Martini, C. (2015) Targeting the 18-kDa translocator protein: recent perspectives for neuroprotection. *Biochem. Soc. Trans.*, **43**, 559–565.
60. Girard, C., Liu, S., Adams, D., Lacroix, C., Sineus, M., Boucher, C., Papadopoulos, V., Rupprecht, R., Schumacher, M. and Groyer, G. (2012) Axonal regeneration and neuroinflammation: roles for the translocator protein 18 kDa. *J. Neuroendocrinol.*, **24**, 71–81.
61. Gong, J., Szego, E.M., Leonov, A., Benito, E., Becker, S., Fischer, A., Zweckstetter, M., Outeiro, T. and Schneider, A. (2019) Translocator protein ligand protects against neurodegeneration in the MPTP mouse model of parkinsonism. *J. Neurosci.*, **39**, 3752–3769.
62. Alam, M.M., Lee, J. and Lee, S.Y. (2017) Recent progress in the development of TSPO PET ligands for neuroinflammation imaging in neurological diseases. *Nucl. Med. Mol. Imaging*, **51**, 283–296.
63. Best, L., Ghadery, C., Pavese, N., Tai, Y.F. and Strafella, A.P. (2019) New and old TSPO PET radioligands for imaging brain microglial activation in neurodegenerative disease. *Curr Neurol Neurosci Rep*, **19**, 24.
64. Rushworth, G.F. and Megson, I.L. (2014) Existing and potential therapeutic uses for N-acetylcysteine: the need for conversion to intracellular glutathione for antioxidant benefits. *Pharmacol. Ther.*, **141**, 150–159.
65. Aldini, G., Altomare, A., Baron, G., Vistoli, G., Carini, M., Borsani, L. and Sergio, F. (2018) N-acetylcysteine as an antioxidant and disulphide breaking agent: the reasons why. *Free Radic. Res.*, **52**, 751–762.
66. Ezerina, D., Takano, Y., Hanaoka, K., Urano, Y. and Dick, T.P. (2018) N-acetyl cysteine functions as a fast-acting antioxidant by triggering intracellular H_2S and sulfane sulfur production. *Cell chemical biology*, **25**, 447–459 e444.
67. Albrecht, S.C., Barata, A.G., Grosshans, J., Teleman, A.A. and Dick, T.P. (2011) In vivo mapping of hydrogen peroxide and oxidized glutathione reveals chemical and regional specificity of redox homeostasis. *Cell Metab.*, **14**, 819–829.
68. Santos, M.M., Ohshima, K. and Pandolfo, M. (2001) Frataxin deficiency enhances apoptosis in cells differentiating into neuroectoderm. *Hum. Mol. Genet.*, **10**, 1935–1944.
69. Piermarini, E., Cartelli, D., Pastore, A., Tozzi, G., Compagnucci, C., Giorda, E., D'Amico, J., Petrini, S., Bertini, E., Cappelletti, G. *et al.* (2016) Frataxin silencing alters microtubule stability in motor neurons: implications for Friedreich's ataxia. *Hum. Mol. Genet.*, **25**, 4288–4301.
70. Myers, L., Farmer, J.M., Wilson, R.B., Friedman, L., Tsou, A., Perlman, S.L., Subramony, S.H., Gomez, C.M., Ashizawa, T., Wilmot, G.R. *et al.* (2008) Antioxidant use in Friedreich ataxia. *J. Neurol. Sci.*, **267**, 174–176.
71. Port, F., Chen, H.M., Lee, T. and Bullock, S.L. (2014) Optimized CRISPR/Cas tools for efficient germline and somatic genome engineering in Drosophila. *Proc. Natl. Acad. Sci. USA.*, **111**, E2967–E2976.
72. Tricoire, H., Battisti, V., Trannoy, S., Lasbleiz, C., Pret, A.M. and Monnier, V. (2009) The steroid hormone receptor EcR finely modulates Drosophila lifespan during adulthood in a sex-specific manner. *Mech. Ageing Dev.*, **130**, 547–552.
73. Reinhardt, A., Feuillette, S., Cassar, M., Callens, C., Thomassin, H., Birman, S., Lecourtois, M., Antoniewski, C. and Tricoire, H. (2012) Lack of miRNA misregulation at early pathological stages in Drosophila neurodegenerative disease models. *Front. Genet.*, **3**, 226.