The *Drosophila junctophilin* gene is functionally equivalent to its four mammalian counterparts and is a modifier of a Huntingtin poly-Q expansion and the Notch pathway

Eduardo Calpena\(^1\)\(^a\), Víctor López del Amo\(^1\), Mouli Chakraborty\(^2\), Beatriz Llamusí\(^2\), Rubén Artero\(^2\), Carmen Espinós\(^1\),\(^4\),\(^#\) and Máximo I. Galindo\(^1\),\(^3\),\(^4\),\(^#\)

\(^1\)Program in Molecular Mechanisms of Disease, Centro de Investigación Príncipe Felipe (CIPF), c/ Eduardo Primo Yúfera no. 13, 46012 Valencia, Spain.

\(^2\)Translational Genomics Group, Incliva Health Research Institute, Avda. Menendez Pelayo 4 acc 46010, Valencia, Spain; Department of Genetics and Estructura de Recerca Interdisciplinar en Biotecnologia i Biomedicina (ERI BIOTECMED), Universitat de València, c/ Dr Moliner 50, 46100 Burjasot, Spain

\(^3\)Instituto Interuniversitario de Investigación de Reconocimiento Molecular y Desarrollo Tecnológico (IDM), Universitat Politècnica de València, Universitat de València, 46022 Valencia, Spain.

\(^4\)UPV-CIPF Joint Unit Disease Mechanisms and Nanomedicine

\(^a\) present address: Clinical Genetics Group, Weatherall Institute of Molecular Medicine, University of Oxford, Oxford OX3 9DS, United Kingdom.

\(^#\) These authors have contributed equally.

Corresponding author: Máximo I. Galindo. email: igalindo@cipf.es

**Keywords**

*Junctophilin, Drosophila, Cardiomyopathy, Huntington disease, Notch*
SUMMARY STATEMENT

This work reveals that the *Drosophila* Junctophilin protein has similar functions to its mammalian homologs; and in addition uncovers new interactions of potential biomedical interest with Huntingtin and Notch signalling.

ABSTRACT

Members of the Junctophilin (JPH) protein family have emerged as key actors in all excitable cells with critical implications for human pathophysiology. In mammals this family consists in four members (JPH1-4) that are differentially expressed throughout excitable cells. The analysis of knockout mice lacking JPH subtypes has demonstrated their essential contribution to physiological functions in skeletal and cardiac muscles, and neurons. Moreover, mutations in the human JPH2 gene are associated with hypertrophic and dilated cardiomyopathies; mutations in JPH3 are responsible for the neurodegenerative Huntington’s disease-like-2 (HDL2), whereas JPH1 acts as a genetic modifier in CMT2K peripheral neuropathy.

*Drosophila melanogaster* has a single *junctophilin (jp)* gene, as it is the case in all invertebrates, which may retain equivalent functions of the four homologous JPHs genes present in mammalian genomes. Therefore, due to the lack of putatively redundant genes, a *jp* *Drosophila* model could provide an excellent platform to model the Junctophilin-related diseases, to discover the ancestral functions of the JPHs and to reveal new pathways. By up- and down-regulation of Jp in a tissue-specific manner in *Drosophila*, we show that altering its levels of expression produces a phenotypic spectrum characterized by muscular deficits, dilated cardiomyopathy and neuronal alterations. Importantly, our study has demonstrated that Jp modifies the neuronal degeneration in a *Drosophila* model of Huntington's disease, and it has allowed us to uncover an unsuspected functional relationship with the Notch pathway.
Therefore, this *Drosophila* model has revealed new aspects of Junctophilin function that can be relevant for the disease mechanisms of their human counterparts.

**INTRODUCTION**

Junctophilin (JPH) family proteins contribute to the formation and maintenance of junctional membrane complexes (JMC) by serving as a physical bridge between the plasma membrane (PM) and the endoplasmic reticulum (ER) membrane in excitable cells, allowing the functional crosstalk between ion channels (Takeshima et al., 2015; Takeshima et al., 2000). Silencing or genetic ablation of the JPHs produces defects in Ca\(^{2+}\) homeostasis (Hirata et al., 2006; Ito et al., 2001; Li et al., 2010; Moriguchi et al., 2006; Takeshima et al., 2015; Takeshima et al., 2000). In mammals, this family comprises four members (JPH1-4) that are differentially expressed: *JPH1* is predominantly expressed in skeletal muscle, *JPH2* in skeletal muscle and heart, and *JPH3* and *JPH4* genes are co-expressed in neural tissues (Nishi et al., 2000; Nishi et al., 2003; Takeshima et al., 2000).

*Jph1* knock-out mice exhibit suckling failure and die shortly after birth with morphological and physiological abnormalities in skeletal muscle, including fewer JMCs, failure of normal triad development, abnormal ER features and reduced contractile force (Ito et al., 2001; Komazaki et al., 2002). *Jph2* plays a key role in cardiomyocyte development and stability of myocyte ultrastructure (Beavers et al., 2014; Chen et al., 2013; Reynolds et al., 2013; Takeshima et al., 2000). *Jph2* null mice die during the early embryonic development due to cardiac failure (Takeshima et al., 2000). Cardiac myocytes from these mutant mice showed deficiency of the JMCs and abnormal structures in ER and mitochondria. Mice with decreased levels of cardiac *Jph2* showed defective postnatal T-tubule maturation, whereas mice over-expressing *Jph2* had accelerated T-tubule maturation (Reynolds et al., 2013). Inducible cardiac-specific *Jph2* knockdown in mice leads to ventricular dilation, postnatal heart failure, and increased mortality (Reynolds et al., 2013).
null mice exhibit adult onset, progressive motor dysfunction, whereas hemizygous mice have a similar but milder phenotype (Seixas et al., 2012). Knockout mice lacking show no obvious abnormalities, suggesting functional redundancy between Jph3 and Jph4 (Moriguchi et al., 2006). Double-knockout mice lacking both Jph3 and Jph4 genes have severe growth retardation and die within 3-4 weeks after birth, probably due to impairment of the neuronal circuit controlling the salivary gland (Moriguchi et al., 2006). In addition, they exhibited impaired motor coordination, learning and memory (Ikeda et al., 2007; Kakizawa et al., 2008; Moriguchi et al., 2006).

genes have been found to play important roles in pathology. In an mdx mouse model of Duchenne muscular dystrophy aberrant proteolysis was detected, providing an association with the development of primary muscle disease (Murphy et al., 2013). JPH2 dysregulation has been associated with variety of heart diseases (Landstrom et al., 2014; Takeshima et al., 2015). Decreased levels of Jph2 expression have been reported in animal models of aortic stenosis (Xu et al., 2007) and hypertrophic and dilated cardiomyopathy (Minamisawa et al., 2004). In addition, in human failing heart samples or in patients with hypertrophic cardiomyopathy, the levels are markedly reduced (Landstrom et al., 2011; Zhang et al., 2013). Importantly, dominant mutations in the human gene are associated with hypertrophic and dilated cardiomyopathy (Beavers et al., 2013; Landstrom et al., 2007; Sabater-Molina et al., 2016) and constitute a relatively rare cause of congenital cardiomyopathies.

Huntington disease-like 2 (HDL2) is a rare, autosomal dominant neurodegenerative disorder that is clinically nearly indistinguishable from Huntington disease (HD). HDL2 is caused by a CTG/CAG expansion located within the alternatively spliced exon 2A of the gene (Holmes et al., 2001). The pathogenicity of this mutation involves both a toxic gain of function due to the expansion and a reduction in the levels of JPH3 protein expression (Seixas
et al., 2012). Recently, *JPH1* has been described as a genetic modifier for the Charcot-Marie-Tooth 2K (CMT2K) peripheral neuropathy (Pla-Martín et al., 2015).

*Drosophila melanogaster* has a single *junctophilin (jp)* gene, as it is the case in all invertebrate genomes (Garbino et al., 2009; Takeshima et al., 2015) which can be an advantage to study the molecular function of this protein family. In addition to the power of genetic analysis in *Drosophila*, the presence of a single *jp* gene will prevent masking of the mutant phenotype by other family members as it has been described for murine *Jph3* and *Jph4*. The restricted tissue specificity of the different JPH genes in mammals, is not completely clear-cut: JPH1 is required in peripheral nerves in addition to its more studied role in muscle (Pla-Martín et al., 2015); and the neural JPH3 and JPH4 proteins are also required in pancreatic β cells and T cells respectively (Li et al., 2016; Woo et al., 2016). Therefore, an animal model with a single *junctophilin* gene can help to uncover more ancestral functions.

We decided to investigate the phenotypic spectrum of altering *jp* in *Drosophila* in order to find out whether they also reproduce histological alterations compatible with those found in knockout mice lacking *Jph* subtypes and in patients with mutations in *JPH* genes; and to reveal new aspects of Junctophilin function that can be relevant for the disease mechanisms of their human counterparts.
RESULTS

Generation of the over-expression and RNAi models

We decided to generate models for over-expression (OE) and knock-down (KD) of \(jp\) to investigate their effects on the target tissues. Both conditions are based on the Gal4/UAS system for directed expression (Brand and Perrimon, 1993), where a tissue-specific Gal4 construct drives expression of another transgenic construct under the UAS promoter.

The single *Drosophila junctophilin* gene (\(jp\), initially annotated CG4405) is homologous to the four human \(JPH\) genes (\(JPH1-4\)), which originated from a single ancestral gene by successive duplications (Garbino et al., 2009). High-throughput studies in *Drosophila* have shown that \(jp\) is expressed in both muscular and neural tissues (Chintapalli et al., 2007; Kumar et al., 2011), data available from http://flybase.org. The \(jp\) gene produces five different transcripts that differ in their transcription start sites, four of which produce the same 1054-aa-long open reading frame coding for the canonical Jp protein (Fig. 1A). The fifth transcript, coding for a 129-aa-long ORF, is probably spurious and definitely non-functional since it lacks most functional. To generate a UAS-\(jp\) we obtained a stock with an insertion of the \(P\{XP\}\) transgene within the \(jp\) locus, \(P\{XP\}jp^{d04563}\). This \(P\{XP\}\) transgenic construct is inserted upstream of the first coding exon (Fig. 1A) and has two UAS promoters, one at each end and in different orientations, so the right promoter points towards the \(jp\) gene and the left promoter towards the upstream region (Thibault et al., 2004). This second promoter is flanked by \(FRT\) recombination sites, so it was removed by crossing to a \(FLP\) transgene, leaving only the \(jp\)-specific UAS promoter, details of this process are given in Fig. S1. This insertion will be referred to as UAS-\(jp\). An advantage of this UAS line compared to the ones generated by random insertion is that, since it is already inserted in the target locus, it does not produce insertional mutations in other genes. Since the insertion is viable in homozygosis, most likely it does not affect expression of the \(jp\) gene substantially, but we cannot discard that it partially
hinders expression and therefore it could be a mild hypomorphic allele. In order to test this possibility we quantitated the \textit{jp} mRNA levels in individuals heterozygous and homozygous for the insertion. This analysis revealed no statistically significant differences with \textit{Oregon-R} wild type flies (Fig. 1B), so the insertion does not affect \textit{jp} transcript expression levels and therefore it is not a hypomorph.

For RNAi we obtained a stock from the Vienna \textit{Drosophila} Resource Centre collection (Dietzl et al., 2007) that contains the insertion \textit{P\{KK107921\}VIE-260B}, whose target sequence is contained in an exon which is common to the four major isoforms of the \textit{jp} gene (Fig. 1A). This line is described as having one on-target and no off-target sites, and has the maximum s\textsubscript{19} specificity score of 1 (Dietzl et al., 2007) and only 3 CAN repeats, which is below the desired threshold of 6 repeats (Ma et al., 2006). In addition, the KK collection was generated by insertion of the \textit{UAS-RNAi} constructs into fixed attP sites in order to achieve reproducible expression levels and also avoid position effects and random insertional mutagenesis. This insertion will be referred to as \textit{UAS-jp\textsuperscript{RNAi}}.

To validate both constructs, we crossed them to different \textit{Gal4} lines that drive expression ubiquitously (\textit{Ac5C-Gal4/CyO}) or specifically in muscular (\textit{twi;Mef2-Gal4 (II)}, early mesoderm and derivatives; \textit{Mhc-Gal4 (II)}, differentiated muscle) or nervous tissues (\textit{elav-Gal4/CyO}, post-mitotic neurons). Since the \textit{twi;Mef2-Gal4} and \textit{Mhc-Gal4} insertions are homozygous viable, if the cross with one of the \textit{UAS} lines was lethal we would observe no progeny, making it difficult to assess whether this was true lethality or due to a crossing failure. For this reason we used both \textit{UAS} lines over a \textit{CyO} chromosome, even in the cross were lethal we should observe several \textit{CyO} flies.

All four gave a viable progeny when crossed to \textit{UAS-jp}, and so did the combination of \textit{UAS-jp\textsuperscript{RNAi}} with the late drivers \textit{Mhc-Gal4} and \textit{elav-Gal4}; but the combination of \textit{UAS-jp\textsuperscript{RNAi}} with early expression drivers \textit{Ac5C-Gal4} and \textit{twi;Mef2-Gal4} was lethal (Figure 1C). This suggests
that the *jp* gene is strictly required for development and/or cell survival. We tried other ubiquitous lines and lower culture temperatures but all attempts resulted in lethality during embryonic or early larval development.

Next, we tested that the *UAS-jp* and *UAS-jpRNAi* lines had the expected effect on the levels of *jp* transcript, by means of quantitative PCR. With the strongest line, *Act5C-Gal4*, we obtained a five-fold increase in *jp* transcript levels with the *UAS-jp* construct (Fig. 1D), but since the cross to *UAS-jpRNAi* was lethal, we could not perform the quantitation in this condition. To validate the *UAS-jpRNAi* construct we dissected thoraxes of *Mhc-Gal4/*UAS-jpRNAi, where muscle tissue is predominant, and we observed a significant decrease in *jp* levels (Fig. 1D). This decrease is an underestimation of the real one since the thorax contains other tissues where Mhc-Gal4 is not expressed such as the thoracic ganglion of the central nervous system. Further proof of the specificity of this RNAi construct is the fact that it can compensate for over-expression of *jp* in different tissues (see below). We used these *Drosophila* transgenic lines to analyse the effect of altering the levels of Jp in tissue-specific OE and KD conditions, by comparing to control flies bearing the same *Gal4* driver but no UAS construct.

**Muscular deficits in the *Drosophila* Jp models**

In mammals JPH1 is the major JPH family member expressed in skeletal muscle (Ito et al., 2001; Komazaki et al., 2002). In *Drosophila*, as we mentioned above, silencing of *jp* with the early mesoderm-specific driver *twi;Mef2-Gal4* resulted in lethality, so we had to use the muscle-specific *Mhc-Gal4* driver that produced viable adults (Fig. 1B). Our control genotype was *Mhc-Gal4/*+, the OE genotype *Mhc-Gal4/UAS-jp*, and the KD genotype *Mhc-Gal4/UAS-jpRNAi*. Regarding longevity, OE flies had only a slight reduction in viability, while KD flies had an extension of the maximum lifespan of around 10 days (Fig. 2A). The possible role
of insulin signalling down-regulation in this extended lifespan is discussed below. In order to measure the muscular competence we performed negative geotaxis and flight assays (Fig. 2B, B’). In both tests the KD flies already showed a markedly decreased performance at 1 week of age; and both OE and KD genotypes had a more severe age-dependent reduction in the ability to climb the vial and to attain stable flight.

To determine whether this motor deficit is due to muscular degeneration we analyzed the indirect flight muscles (IFM) in semi-thin sections of the thorax. No significant differences were found at one week of age, but at four weeks horizontal gaps within each set of muscle packs are evidently reduced in KD flies compared to control and OE flies (Fig. 2C-C’’), and this is due to an increase in the area of the IFM sections (Fig. 2 D). This result suggests an age-dependent muscle hypertrophy in KD flies which may be related to the defects observed in the climbing assay. To study the ultra-structure of IFM by transmission electron microscopy we performed longitudinal sections along the muscle fibres. The structure of the myofibrils was not affected in any genotype, but KD flies displayed an aberrant mitochondrial morphology which was most evident at four weeks (Fig. 2 E-E’’). These mitochondria were smaller and more rounded than in the wild type control. Mitochondria from control and OE flies are similar, with a circularity index around 0.6; while KD mitochondria are more circular, with an average index of 0.75, and these differences are statistically significant.

Since KD flies have abnormally shaped mitochondria, we estimated mitochondrial biomass by qPCR, to determine the mitochondrial genomic DNA content, using the nuclear genomic DNA for normalisation, and we did not find any significant differences between OE or KD and the control.

Since loss of Jph1 in skeletal muscle results in aberrant triads, we examined the morphology of the equivalent structure in the insect muscle, the diads, formed by a single electron-dense SR cisterna and the adjoining electron-lucent T-tubule (Razzaq et al., 2001). We performed
this analysis at 1 week of age, before major morphological changes in mitochondria. Diads are located half-way between the Z and M bands of the myofibril (Fig. 2G). OE muscles have aberrant diads with elongated SR cisternae, and in mitochondria-poor regions unusually elongated SR cisternae are surrounded by electron-lucent material, which may be an expanded T-tubule structure (Fig. 2H). Occasionally, diads also show an abnormal positioning away from the myofibrils and embedded in the mitochondria. Diads in KD muscles have a different phenotype, they are rudimentary or have an aberrant morphology, including a vacuolated SR similar to the one in Jph1 KO mice (Fig. 2I); and the adjoining mitochondrial regions also have abnormal cristae morphology.

Human JPH1 is a modifier of the GDAP1 mutations causing Charcot-Marie-Tooth peripheral neuropathy. (Pla-Martin et al., 2015). Previous work from our group found metabolic alterations in Drosophila Gdap1 models (Lopez Del Amo et al., 2017). To determine whether altered levels of Jp result in metabolic changes in the muscle, we carried out a metabolomic study by nuclear magnetic resonance (NMR). In order to detect early and direct metabolic alterations which could contribute to reduced muscular competence, we performed this study in 1-week-old flies. We compared the control genotype to the OE and KD genotypes, and in both cases we could find a discriminating model by orthogonal projection on latent structure-discriminant analysis (OPLS-DA) (Fig. S2), which means that the compared genotypes are metabolically different. The most marked change in both experimental genotypes is a significant increase in glycogen, the main carbohydrate storage species in the muscle. At the same time, there is a reduction in trehalose, which is the main source of energy for the IFM. Next, we paid attention to metabolites known to reflect the homeostasis of the muscle. β-alanine abundance is a marker of muscle degeneration (Sarou-Kanian et al., 2015), and levels of this metabolite had no significant changes. The three branched-chain aminoacids (isoleucine, leucine and valine) promote protein synthesis in the muscle (Kimball and Jefferson, 2006), and their abundance was reduced only in KD flies. These results reinforce
the notion that imbalanced \textit{jp} levels affect muscle function but do not result in major muscular degeneration.

\textbf{Cardiac dysfunction produced by altered Jp expression}

Constitutive or heart-specific loss \textit{Jph2} produces cardiac defects in mice (Takeshima et al., 2000). In order to investigate the relevance of \textit{Jp} in the \textit{Drosophila} heart we used the cardiac-specific driver \textit{GMH5-Gal4}. All the results we show below have been obtained with a culture temperature of 29°C, at which the cardiac phenotypes were most evident. In this case, our control genotype was \textit{GMH5-Gal4/+}, the OE genotype \textit{GMH5-Gal4/UAS-jp}, and the KD genotype \textit{GMH5-Gal4/UAS-jp\textsuperscript{RNAi}}. Both, interference and over-expression of \textit{jp} caused a reduction of lifespan from 53 days in control flies to only 35 days (Fig. 3A). Mean survival was also reduced from 47 days in control flies to 27 days in OE flies and only 17 days in KD flies. In this survival curve it is evident that the lifespan of the control flies is very reduced compared to other survival curves in this work (Fig 2A, Fig4A below). This reduction is due to culture at 29°C, which is a sub-optimal temperature but allows a better development of cardiac phenotypes.

To study heart function, cardiac contractions were analysed in 1-week-old adult fly hearts. There was no alteration of the diastolic interval (DI), the systolic interval (SI) or heart period length (HP, defined as DI + SI) (Fig. S3). The arrhythmia index (AI), an indicator of the variability calculated by dividing the standard deviation of the heart period by its median was also unaltered (Fig. S3).
In contrast, we found changes in cardiac chamber parameters, including increased end systolic diameter (ESD) and end diastolic diameter (EDD) and decreased fractional shortening percentage (FS), which provides an indication of the cardiac output (Fig. 3B-B’’). These changes are evident in the M-mode traces of the three genotypes (Fig 3C). These data revealed that in flies with altered levels of Jp expression heart tube is dilated and there is a dysfunction of the contractile properties which reduces cardiac output. For a more detailed observation of heart morphology in the \(jp\) mutants, we performed transversal semi thin sections of the heart tube (Fig. 3D-D’’). We observed and enlargement of cardiac chamber, which we have previously quantified as an increased end diastolic diameter (Fig. 3B’). Notably, these sections show that the thickness of heart walls in the mutant flies do not show any statistically significant differences to control flies, discarding hypertrophy of cardiomyocytes (Fig. 3E).

*Drosophila* heart tubes have two types of muscle fibres, each with a distinct myofibrillar structure (Mery et al., 2008; Tagli-Lamallem et al., 2008); spirally or transversely oriented myofibrils that represent the contractile ‘working’ myocardium, and longitudinally oriented myofibrils that are found along the ventral surface of the tube (Molina and Cripps, 2001). In young flies, both types of myofibrils exhibit a tight and well-aligned arrangement. Cardiac myofibrils stain uniformly along the entire length of the thin filament with phalloidin (Ao and Lehrer, 1995) so it can be used to visualize both types of myofibrils. Phalloidin staining of actin in mutant flies with altered expression of \(jp\) revealed structural abnormalities in the parallel alignment of transverse myofibrils in the heart tube in the areas surrounding the ostia (Fig. 3D-D’’). The cardiac fibres in the mutant flies were clearly more disorganized and less compact than in control flies. The gaps in myofibrillar staining were quantified by measuring the size of these areas in confocal stacks of five hearts of each genotype (Fig 3G). The
percentage area devoid of myofibrils was significantly smaller in control hearts compared to that of KD or OE, 2%, 10% and 7.8% respectively.

**Modification of the Jp levels produces neurological abnormalities and affects the number of photoreceptor neurons**

To evaluate the neuronal relevance of Jp in *Drosophila* we used the pan-neuronal *elav-Gal4* driver, the control genotype was *elav-Gal4/+*, the OE genotype *elav-Gal4/UAS-jp*, and the KD genotype *elav-Gal4/UAS-jp<sup>RNAi</sup>*. Mean survival was strongly reduced in OE flies whereas a slightly reduction was observed in the KD flies (Fig. 4A). The bang-sensitive phenotype, a temporary paralysis when exposed to mechanical stress, has been associated with mutations in genes involved in neuronal function (Graham et al., 2010; Kuebler and Tanouye, 2000; Lee and Wu, 2002; Pavlidis et al., 1994; Schubiger et al., 1994; Trotta et al., 2004). Bang-sensitivity analyses revealed that KD flies exhibit a 2- to 3-fold increase in recovery time at 1 week and at least a 5-fold increase at 4 weeks compared with control flies (Fig. 4B). The recovery time of the OE flies was also increased, and the difference was statistically significant at 4 weeks.

The fly retina is a widely used tissue to study neurodegeneration. To drive expression in the retina we used the *GMR-Gal4 (I)* construct (control genotype *GMR-Gal4/+*, OE genotype *GMR-Gal4/+; UAS-jp/+*, and the KD genotype *GMR-Gal4/+; UAS-jp<sup>RNAi</sup>/+*). In the control eyes we can observe the wild type external morphology, the ommatidial lenses are dome-shaped and arranged in a hexagonal tiling pattern with inter-ommatidial bristles (Fig 4C, C’). Over-expression of Jp produces a mildly disrupted arrangement, the presence of non-hexagonal ommatidia and supernumerary bristles (Fig 4D, D’). In contrast, KD resulted in no observable abnormalities in the external morphology (fig. 4E, E’). Although *GMR-Gal4; UAS-jp<sup>RNAi</sup>* on its own has no phenotype, it is able to correct the phenotype of the eye external
morphology caused by over-expression of \textit{UAS-}jp under the control of \textit{GMR-Gal4}, which demonstrates the specificity of the abnormal phenotype due to the over-expression of \textit{jp} (Fig. 4F, F’).

A cross section of a normal ommatidium always cuts through seven rhabdomeres, the light-collecting organs of the neuron, in a stereotypical trapezoidal arrangement (Fig 4G). Transmission electron microscopy analyses of OE and KD retinas revealed abnormal number of photoreceptors in both genotypes (Fig. 4G-J). Almost 50\% of the OE ommatidia had extra photoreceptor cells, whereas in KD flies the effect in the opposite, with several ommatidia containing fewer photoreceptor cells (Fig. 4I). The presence of extra photoreceptor cells has been extensively described as a consequence of signalling defects during the development in the eye, whereas loss of photoreceptor could be due to defects in such signalling pathways but also due to neurodegenerative processes even at early post-eclosion stages, or a mixture of them. To discriminate between these situations we performed the following experiment. To reduce the expression of the \textit{jp RNAi} during development, flies were crossed and reared at 18°C until eclosion. At this point the flies were divided into two groups; one was kept for 1 day at 18°C the other was cultured for 7 days at 25°C after eclosion to allow stronger expression of the RNAi. In KD flies that were kept at 18°C the morphology is slightly compromised probably as a result from an incipient degeneration since RNAi expression is damped, not abolished, but at this point most ommatidia have 7 photoreceptors. KD flies that were moved to 25°C have an enhanced loss of photoreceptors and a more degenerative morphology with many vacuoles (Fig. S4). Therefore, while over-expression of \textit{jp} results in recruitment of extra photoreceptor neurons, \textit{jp RNAi} results mainly in neurodegeneration rather than photoreceptor specification.
The Htt-related neurodegeneration is modified by altering the Jp levels

Dominant mutations in the \textit{JPH3} gene caused by an expanded CAG/CTG repeat in its alternatively spliced exon 2A are responsible for the Huntington disease-like 2 (HDL2), a phenocopy clinically indistinguishable from Huntington disease (HD) (Holmes et al., 2001). HD is a fatal neurodegenerative condition caused by expansion of the polyglutamine tract in the Huntingtin (Htt) protein, and the precise disease manifestations and their timing are affected by modifier genes (Gusella and MacDonald, 2009). Despite the phenotypical similarities, a possible role of JPHs as genetic modifiers in HD has not been investigated.

HD has been modelled several times in \textit{Drosophila} by over-expression of pathological expansions under neuronal drivers (Lewis and Smith, 2016), and these models have similar phenotypes to ours: reduced lifespan when expressed under \textit{elav-Gal4} and retinal neuron degeneration under \textit{GMR-Gal4} (Fig. 4); and in addition, they also show features typical of HD such as protein aggregates. The abnormal phenotypes in these models are repeat-length-dependent. To model HD in \textit{Drosophila}, we used a construct for the expression of human \textit{HTT} exon 1 containing expanded poly-glutamine repeats (\textit{Htt-Ex1-pQ93}) that has been demonstrated to induce neurodegeneration (Steffan et al., 2001). These authors describe that shorter repeats (1Q or 20Q) have no deleterious effect. In order to investigate whether Jp could modify the HD pathogenesis in flies, \textit{UAS-}j\textit{p} or \textit{UAS-jp^{RNAi}} were co-expressed with \textit{Htt-Ex1-pQ93}. Flies expressing \textit{Htt-Ex1-pQ93} have a normal external morphology of the eye at day 1 post-eclosion, but suffer progressive degeneration of the underlying retina resulting in patchy de-pigmentation after four weeks of age (Fig. 5A-A’’). When \textit{jp} is co-expressed with \textit{Htt-Ex1-pQ93} the development of this de-pigmentation is delayed (Fig 5B-B’’), which means that Jp may act as a partial suppressor of the \textit{Htt-Ex1-pQ93}-induced degeneration.

Conversely, co-expression of \textit{Htt-Ex1-pQ93} with the \textit{jp RNAi} led to enhancement of eye phenotype, since the loss of pigmentation is observed from day 1, and the de-pigmentation...
progresses much faster (Fig 5C-C‘‘). As a control experiment, we aged flies of the GMR-Gal4/+ , GMR-Gal4; UAS-jp and GMR-Gal4; UAS-jpRNAi and observed no changes in pigmentation, so the results observed were bona fide genetic modifications.

The Htt-Ex1-pQ93 corresponds to a truncated version of the gene coding for just a few endogenous aminoacids, so it is possible that function \( jp \) as a modifier for other types of pathogenic poly-Q expansions. To test this possibility we investigated whether \( UAS- jp \) or \( UAS- jpRNAi \) were able to modify the phenotype caused by expression of another dominant mutation caused by a poly-Q expansion in the human SCA3 gene causative of spinocerebelar ataxia type 3 (Stochmanski et al., 2012). \( UAS-SCA3-Q89 \) contains an expanded tract of glutamines within a full-length SCA3 cDNA, and it also causes de-pigmentation of the Drosophila retina when expressed under GMR-Gal4 (Fig. 5G). Again co-expression of \( UAS- jp \) slows down de-pigmentation at 4 weeks (Fig. 5H) and co-expression of \( UAS- jpRNAi \) enhances it (Fig 5I). The modification of the phenotype is clear, suggesting that mechanistically \( jp \) can be a modifier of poly-Q expansions in general, but this does not necessarily mean that the modification is clinically relevant since SCA3 and HD/HDL2 affect different regions of the encephalon.

**Jp has functional interactions with the Notch pathway**

A closer examination of the normally viable Act5C-Gal4/UAS-jp individuals revealed abnormal phenotypes usually linked to a down-regulation of the Notch signalling pathway such as supernumerary vibrissae under the eye, microchaetae in the notum and sternopleural bristles (Fig. 6A, B) (Schweisguth and Posakony, 1994). Further Notch phenocopies are the duplication of the macrochaetae in the scutellum (Fig 6C) and delta-shaped wing veins (Figure 6D). All these phenotypes were 100% penetrant. The phenotype we observed under OE of \( jp \) in the retina also points out to a down-regulation of Notch signalling, since the
recruitment of extra cells as photoreceptor neurons is typical of mutants for the Notch ligand Delta (Parks et al., 1995). If this was a result of a functional interaction between Jp and the Notch pathway, either direct or indirect, the prediction would be that \( \text{jp OE} \) should enhance Notch phenotypes and \( \text{jp KD} \) should suppress them. To carry out these tests we could not use the \( \text{Act5C-Gal4} \) driver with which we detected the phenotypes since \( \text{jp KD} \) with this driver is lethal before the adult age. We also discarded the retina as an experimental tissue since Notch signalling has successive and complex roles during eye development, including growth, planar cell polarity and several rounds of cell-type specification (Cagan and Ready, 1989).

The wing blade is a more suitable model to investigate modification of the Notch/Delta phenotypes, so we used two \( \text{Gal4} \) lines that drive expression in the whole wing blade, \( \text{rn-Gal4} (\text{III}) \) and \( \text{nub-Gal4} (\text{II}) \).

First we tested the effect of \( \text{jp OE} \) and KD on their own. OE of \( \text{jp} \) driven by \( \text{rn-Gal4} \) driver again mimics a Notch phenotype: thickening of the longitudinal wing veins and delta-shaped contacts of the veins and the wing margin (De Celis, 2003); and also produce a reduction of the wing area (Fig. 6E, F, H). In contrast, \( \text{jp KD} \) did not produce any evident phenotypes in the wing morphology (Fig 6E, G, H). Similar experiments with the other wing driver, \( \text{nub-Gal4} \), produced equivalent results and also showed that \( \text{jp KD} \) can correct \( \text{jp OE} \) despite not having an effect on its own (Fig 5S). To test whether the extra vein tissue was produced by a reduction in the activation of the Notch pathway, we attempted the modification of the phenotype of a dominant temperature-sensitive allele of the gene encoding the Notch ligand Delta, \( \text{Dl^{6B37}} \). These experiments were performed at 29ºC, the temperature at which this \( \text{Dl} \) allele shows a more pronounced phenotype than in the normal rearing conditions at 25ºC. In these conditions, again OE wings have a \( \text{Dl} \)-like phenotype and KD wings are normal (Fig 7A, B, C). \( \text{Dl^{6B37}} \) flies displayed the expected wing vein defects, which are most evident in the L2 vein, and delta-shaped contacts with the wing margin (Fig7D). Expression of Jp in the wing blade enhanced these phenotypes (Fig 7E), while expression of the \( \text{jp RNAi} \) corrected
them (Fig 7F). In order not have a parameter that allowed for a statistical treatment we measured the length of the contact of L2 with the wing margin, which is wider when it adopts the delta shape. These analyses confirmed that \( jp \) behaves as it would be expected from a typical modifier: OE of \( jp \) enhances the \( Dl \) phenotype and KD partially suppresses it (Fig 7G).

**DISCUSSION**

*Drosophila as a model to study Junctophilin function.*

In the present work we present data indicating that *Drosophila* is a good model to study pathologies resulting from mutations in human *JPH* genes. Our two experimental genotypes are based on a gain and a loss of function, OE and KD respectively. In general, the specific phenotypes we observe in a particular tissue are also opposite (i.e. the effect on Htt expansions or Notch wing phenotypes), although some of the non-specific phenotypes such as overall viability or motility can be altered in the same sense in both. This reduced fitness indicates that a proper balance in the expression levels of \( jp \) is required for normal calcium homeostasis cell functions. The phenotypes we have described in muscle, heart and neurons are compatible with what has been described in patients with pathological mutations in *JPH* genes or in KO mouse models of these genes. In addition, we describe two novel functional relationships that may be relevant in the pathogenesis of junctophilins with pathological poly-Q expansions in Huntington disease and with Notch signaling.
**Junctophilin function in the heart and muscle.**

Down- and up-regulation of *jp* expression affects mainly the structure of the diads in the IFMs. Elevated levels induce the formation of elongated SR cisternae and, less often, incorrect localization of the diad next to the myofibrils. This effect is similar to the one observed upon elevated expression of *Jhp1* in mouse heart (Komazaki et al., 2003). In these mice, cardiac diads had extended SR-T-tubule contacts. Overexpression of Jph2 in heart also increases the SR-T-tubule contacts (Guo et al., 2014). KD of *Drosophila* *jp* also produced phenotypes comparable to those observed in Jph1 KO mice: incorrect formation of diads and vacuolated SR and T-tubules. The phenotypes in KD flies are dramatic, with rudimentary or nearly absent diads at 1 week and a fragmentation of the mitochondrial network at four weeks. This fragmentation in older flies could be related to the incipient mitochondrial damage observed in younger ones. In murine muscle there is co-expression of Jph2, so our KD phenotype probably represents a more severe loss of function, thus unveiling that junctophilins are required for the proper formation of the SR-T-tubule contacts, not only their maturation.

In any case, neither our experimental genotypes nor the published KO or over-expression murine models show any structural degeneration of the myofibrils. This is confirmed in the metabolomic profile, since there were no alterations linked to muscular degeneration, only an imbalance in the carbohydrate levels with increased glycogen and decreased trehalose. This result is confirmed by an independent work, in which a screening for genetic determinants of metabolic traits in whole fly revealed a genetic linkage of *jp* to energy metabolism traits and increased glycogen levels. (Jumbo-Lucioni et al., 2010). A possible explanation for this deregulation of carbohydrate metabolism is an alteration of the insulin signaling pathway.
In fact, down-regulation of the insulin pathway could also explain two unexpected phenotypes in the muscle KD flies: the extended lifespan and the muscle hypertrophy. Mitohormesis, the adaptive response of mitochondria to mild stress, has already been reported to produce an extended lifespan in *Drosophila* (Owusu-Ansah et al., 2013). In this case, mitohormesis triggered by mild muscular stress and mitochondrial fragmentation resulted in lifespan extension, and this effect was mediated by the insulin pathway and the mitochondrial unfolded protein response. There are further examples of extended longevity caused by impairment of insulin signaling and/or mitochondrial stress (Wang and Hekimi, 2015; Zarse et al., 2012). As for muscle hypertrophy, it is usually assumed that low levels of BCAA are indicative of muscle deficiency, but there is a great heterogeneity and many factors impinging at different levels (Tom and Nair, 2006). One of the pathways involved is insulin signaling (Glass, 2005), in this sense we observe significant alterations for glycogen and trehalose levels. This result could be due to the involvement of different pathways and deserves a deeper study.

Muscle hypertrophy was also observed in mouse skeletal muscle expressing Jph2 with the dominant negative mutations S165F and Y141H, associated to hypertrophic cardiomyopathy in patients (Woo et al., 2012; Woo et al., 2010). Several mutations in human JPH2 have been associated to hypertrophic cardiomyopathy (Beavers et al., 2014), and can cause a hypertrophic phenotype when down-regulated in mouse cardiac muscle cell lines (Landstrom et al., 2011). In contrast, the phenotype of the heart KD flies is more similar to a dilated cardiomyopathy, with increased ESD and EDD and reduced cardiac output. Recently, a new mutation in *JPH2* has been found to be associated to dilated cardiomyopathy (Sabater-Molina et al., 2016). Therefore, mutations in *JPH2* could cause hypertrophic or dilated cardiomiopathy depending on factors such as the genetic background, degree of functionality of the mutant protein, or location of the mutation in a particular domain.
A difference between KD in muscle and heart is that in the second instance we could find structural alterations in the cardiac myofibrils. A possible explanation is that the muscular driver we employed, *Mhc-Gal4* is expressed in differentiated muscle; while the cardiac driver, *GMH5-Gal4*, contains a promoter region from the *tinman* gene which drives expression throughout heart development from the early embryo. Alternatively, this difference could be due to intrinsic physiological differences between skeletal and cardiac muscle.

**Junctophilin function in neurons**

A *Jph3* knock-out produces motor deficits in mouse models, and the double knock-out *Jph3/4* has a more severe phenotype including the impairment of motor, learning and memory abilities (Moriguchi et al., 2006; Nishi et al., 2002; Seixas et al., 2012). Similarly, KD of Jp in *Drosophila* neurons also affects neuronal function as reflected in the bang sensitivity test. Alteration of Jp in the retina revealed a mixture of neurodevelopmental defects and degeneration of the retinal neurons. While neurodevelopmental defects probably involve the mis-regulation of the Notch signalling pathway during cell fate determination and differentiation stages, the neurodegeneration happens in fully differentiated neurons and is dependent in neural function or survival rather than development.

The modification of the *Htt-Ex1-pQ93* phenotype by Jp is clear-cut, OE of Jp is a suppressor and KD is an enhancer of the neurodegeneration. This modification is most probably functionally relevant, since ablation of *Drosophila* Htt exacerbates the neural toxicity elicited by the same construct we have used in the present work (Zhang et al., 2009). Also, neuronal store-operated calcium entry is a novel therapeutic target for HD (Wu et al., 2011). Since triplet expansions of JPH3 are causative of HDL2 (Holmes et al., 2001), the fact that altering Jp levels modifies the phenotype of flies expressing human *Htt-Ex1-pQ93* suggests that both proteins participate in at least one common cellular pathway. In addition to causing HDL2, it
is possible that genetic variation in JPH3/4 participates in the clinical variability of HD patients.

A *Drosophila* model of HDL2 based on the expression of the human JPH3 mutant protein HDL2-Q138 showed that toxicity is due to the accumulation of the polyQ-expanded protein in the nucleus and this toxicity was alleviated by re-directing it to the cytoplasm (Krench et al., 2016). In contrast, HTT-Q138 aggregates remain cytoplasmic, which suggests that they do not share toxicity mechanisms. Although this finding may seem to contradict ours, it has been demonstrated that the pathogenicity of *JPH3* mutations is multifactorial and involves at least two effects, a toxic gain of function of the aggregates and a deficit in JPH3 function due to reduced expression levels (Seixas et al., 2012). Therefore, overexpression of the human HTT-Q138 protein and modulation of the endogenous Jp levels could be affecting different cellular mechanisms in the nucleus and the cytoplasm respectively.

**Junctophilin antagonises Notch signalling**

Over-expression of Jp phenocopies the loss of function of the Notch ligand Dl: recruitment of supernumerary photoreceptor neurons, lateral inhibition in sensory organ determination and expansion of the wing veins. A role of Jp as a modifier of Notch is strongly reinforced by the fact that loss of function of Jp can suppress the mutant phenotype of a dominant loss of function allele of Dl. Although this was an unexpected result, there are three previous high-throughput screenings for modifiers of Notch phenotypes in the wing (Cruz et al., 2009; Molnar et al., 2006) or in the eye (Shalaby et al., 2009) in which *Drosophila* *jp* was among the identified candidates. All of them were based on over-expression of genes adjacent to insertions with bi-directional UAS promoters, either the same original insertion we have used in our work (*P{XP}jp*^d04563^) or a similar construct (*P-GS*). In consequence, the authors could not discern which of the two genes flanking the insertion, *jp* or *CG3838*, was the modifier.
Since we have reproduced the modification after removing the promoter pointing to \textit{CG3838}, we can single out \textit{jp} as the Notch-interacting gene.

Our work is the first report of a functional relationship of junctophilins with Notch signaling. The effect of Jp on Notch is most probably through its effect on calcium trafficking. The ER calcium sensor STIM1 has been shown to co-localise with JPH1 during store-operated calcium entry (Pla-Martin et al., 2015) and interacts physically with JPH4 (Woo et al., 2016). \textit{Drosophila Stim} has also been demonstrated to be a modifier of Notch phenotypes (Eid et al., 2008); although in this case \textit{Stim} expression is synergistic rather than antagonistic to the pathway. Another screening in \textit{Drosophila} has unveiled other calcium signaling proteins such as calmodulin or ryanodine receptor as modifiers of presenilin-dependent Notch signaling (van de Hoef et al., 2009).

This intimate relationship of calcium and Notch signaling suggests that defects in Notch could contribute to the pathogenicity of Junctophilin mutations. Disruption of \textit{Drosophila} Notch pathway members results in a dilated cardiomyopathy similar to the one we describe in our models (Kim et al., 2010). As for the neural function of junctophilins, it is remarkable that the phenotypes of two mouse models, the \textit{Jph3/4} double KO and a Notch antisense RNA, have a similar phenotype with impaired synaptic plasticity and long term potentiation in hippocampal CA1 synapses (Moriguchi et al., 2006; Wang et al., 2004). In the light of all this evidence, the interplay of junctophilins and Notch mediated by calcium could prove to be a relevant disease mechanism in muscular and neural pathologies and deserves further attention.
MATERIALS AND METHODS

Drosophila stocks, maintenance and genetics

The following fly stocks were obtained from the Bloomington Drosophila Stock Center: Oregon-R, w^{118}, Df^{6837}, Act5C-Gal4, GMR-GAL4, Mhc-Gal4, Elav-Gal4, nub-GAL4, UAS-Ser, UAS-Dl, UAS-GFP, P(XP)JP^{d04563} and hs-FLP; the RNAi line v100555 expressing a dsRNA for RNAi of j{p} (CG4405) and UAS-Dcr2 were obtained from the Vienna Drosophila Resource Centre. Other drivers used were GMH5-GAL4 (Wessells and Bodmer, 2004), rm-GAL4 (St Pierre et al., 2002), UAS-Htt-ex1-pQ93 (Steffan et al., 2001). twi;Mef-GAL4 is a recombinant carrying both twi-Gal4 and Mef2-Gal4. For the modification of SCA3 expansions we employed UAS-SCA3-Q89, expressing a full length cDNA (Stochmanski et al., 2012).

Flies were maintained on standard cornmeal medium at 25°C unless it is stated otherwise in the Results section.

The UAS-j{p} line was obtained by removing one of the two UAS promoters pointing in opposite directions in the P(XP) transposon in the P(XP)JP^{d04563} insertion, leaving just the UAS promoter pointing towards the j{p} gene. For these, we crossed to flies with that express the Flipase protein under the control of a heat shock promoter, hs-FLP. The removal of the UAS by was confirmed by PCR and sequencing of the amplified fragment. The following oligonucleotides were used for the PCR: JP-CG4405-FLPout-F (TGCTGTGGTCCGTTCTCTTGGC) and JP-CG4405-FLPout-R (TCGGCTGCTCTAAACGACG).
Nucleic acid isolation and qPCR

Quick Fly Genomic DNA Prep protocol (Berkeley Drosophila Genome Project resources) was used to isolate gDNA for genotyping. The methods for the RNA isolation and the cDNA synthesis were previously described (Lopez Del Amo et al., 2015). The qPCRs were performed with SYBR Green SuperMix (Quanta BioSciences, Beverly, MA; USA) in a LightCycler LC480 real-time PCR instrument (Roche, Basel, Switzerland). Each qPCR was performed in triplicate for all genotypes, and each individual sample was obtained by pooling 10 individual flies in the RNA extraction. The relative mRNA levels were calculated according to the $2^{-\Delta\Delta Ct}$ method. Results were normalized to the expression of the Gapdh or Rpl49 housekeeping genes.

For mitochondrial DNA copy number, total DNA was isolated as previously described (Scialo et al., 2015) and analyzed by qPCR using primers against CoxI (for mtDNA) and Rpl32 (nuclear DNA, single-copy, for normalization).

Lifespan and behavioral assays

For lifespan experiments flies were collected using CO$_2$ anaesthesia within 24-48 h of eclosion and then kept at a density of 20-25 flies per vial at 25°C (29°C in the case of the GMH5-GAL4 driver). Flies were transferred to new vials every 2-3 days and the number of dead flies was recorded. Lifespan studies were performed with a minimum of 50 flies from 3 independent experiments.

To examine the locomotor ability the flies were knocked down to the bottom of the vial by quick firm tapping and the proportion of flies that had climbed over the 9 cm mark within 10 seconds was determined. This assay was performed in triplicate for each genotype; at least 15
flies were used per genotype. For the flight assay, individual flies were transferred to a Petri dish, then the lid was removed and the dish inverted over a 45 cm-long cylinder and gently tapped to loosen the fly. Flies that were either able to stabilize their flight and stay at the wall of the vessel, this position was scored in cm, or fell at the base and were scored with 45 cm. 30 flies were scored for each genotype. The bang sensitivity assay was performed as previously described (Graham et al., 2010). A minimum of 10 flies from 4 independent experiments were tested for any particular genotype.

**Cardiac physiological analysis**

For the physiological analysis, female flies were collected just after eclosion and were maintained for 7 days at 29°C. For the heart beat recordings, semi-intact heart preparations and SOHA analysis were made as previously described (Chakraborty et al, 2015; Ocorr et al., 2007). A minimum of 15 hearts were analysed per genotype.

**Histology and microscopy**

Flies were examined under an Olympus SZ60 stereomicroscope (Olympus, Tokyo, Japan) equipped with a Scopetek MDC200 Digital Camera (Hangzhou Scopetek Opto-Electric Co., Hangzhou, China). Areas (retina, IFM and wings) and lengths (L1-L2 wing veins) were measured using the ImageJ software (version 1.47, NIH, Bethesda, MA, USA). Adult cuticles and wings were mounted in Hoyer’s medium and analyzed with a Leica DM6000 microscope. Scanning electron microscopy (SEM) analysis of adult eyes was performed as previously described (Calpena et al., 2015) following the critical point drying method. Images were taken with a Philips XL-30 ESEM scanning electron microscope.
For light and transmission electron microscopy, eyes, thoraxes and abdomens were dissected and fixed overnight with 2% paraformaldehyde and 2% glutaraldehyde in 0.1 M phosphate buffer as previously described (Lopez Del Amo et al., 2015). All samples were post-fixed in OsO₄ for two hours and dehydrated in an ethanol series, and then samples were embedded in Durcupan epoxy resin (Sigma-Aldrich, St. Louis, MO; USA). For transmission electron microscopy, 80 nm thick sections were stained with uranyl acetate and examined with a FEI Tecnai Spirit G2 microscope. For bright field microscopy, 1.5 µm sections of thorax and abdomens were stained with toluidine blue and images were examined with a Leica DM6000 microscope. Thorax sections were used for assessment of muscle defects; for the muscle section area a minimum of six flies per genotype were analysed. Abdomen sections were used to evaluate heart walls thickness, a minimum of 10 sections from different individuals were analysed, and three different measurements of wall thickness were performed in each section. The number of photoreceptor neurons per ommatidium was determined by analysing TEM images of sections of retina from one week flies. For each genotype, sections from three different individuals were studied and at least 60 ommatidia were scored in each section.

For confocal microscopy, fly hearts were dissected from 7-day-old females, fixed for 20 min in 4% paraformaldehyde, washed in PBT (PBS containing 0.3% Triton X-100), and stained with phalloidin for 20 min as previously described (Chakraborty et al., 2015). All confocal images were taken in an Olympus FV1000 microscope. The gaps in myofibrillar staining were quantified by measuring the size of these areas from confocal stacks of five hearts of each genotype using the ImageJ software (version 1.47, NIH, Bethesda, MA, USA). The percentage area devoid of myofibrils was calculated and comparisons were made between the control hearts and the OE or KD genotypes.
Mitochondrial circularity index

For the calculation of the circularity index of the mitochondria, the outline of the mitochondria was manually traced and then analysed with the ImageJ software (version 1.47, NIH, Bethesda, MA, USA). Sample size was three individuals per genotype; from each individual two different muscle electron micrographs were analysed by scoring the index for 30 mitochondria per micrograph (a total of 180 mitochondria per genotype).

NMR spectroscopy

For NMR spectroscopy, six samples were analysed for each one of the three genotypes, each one of them containing 15 flies. Sample preparation, NMR spectroscopy and data analysis were performed as described (Lopez Del Amo et al., 2015).

Statistical analysis.

Data were analyzed with Prism 5 (GraphPad). In the lifespan experiments, log-rank (Mantel-Cox) test was performed and each one of the experimental genotypes was compared to the control. In the comparisons between the three genotypes at a single time point we performed one-way ANOVA with Dunnett’s multiple comparison test. Values shown represent means ± SEM. In all figures *p < 0.05, **p < 0.01, and ***p < 0.001.
ACKNOWLEDGEMENTS

We thank Mario Soriano for his advice with the histology for electronic microscopy and Martina Palomino for her help with the metabolomics analysis; we also thank Alberto Sanz for the mitochondrial DNA quantitation protocol and Hervé Tricoire for sharing fly stocks.

COMPETING INTERESTS

The authors declare that they have no conflict of interest.

AUTHOR CONTRIBUTIONS


FUNDING

This work was supported by project grants from the Association Française contre les Myopathies [AFM 18540 to M.I.G]; from the Instituto de Salud Carlos III (ISCIII) [grants no. PI12/000453 and PI15/000187 to C. E.]; from the Generalitat Valenciana [PROMETEOII/2014/067 to R.A. as partner] and a collaborative grant from International Rare Diseases Research consortium (IRDiRC) and Instituto de Salud Carlos III [IR11/TREAT-CMT to M.I.G. (partner 12) and C.E. (partner 6)]; C.E. has a “Miguel Servet” contract funded by the ISCI-II and the Centro de Investigación Príncipe Felipe [Grant no. CPII14/00002]; M.C. was the recipient of a Santiago Grisolía award from the Generalitat Valenciana [GrisoliaP/2013/A/044]
REFERENCES


not enhance baseline function but attenuates heart failure development after cardiac stress. 

_**Proc Natl Acad Sci U S A**_ 111, 12240-5.  


Figure 1. Generation and validation of stocks for over-expression and RNAi. (A) Schematic representation of the *jp* locus with gene span, structure of the five isoforms identified (coding region light grey), insertion site of the *P[XP]jp^{d04563}* construct (UAS, arrow) and target region of the RNAi line *P(KK107921)VIE-260B* (RNAi, shaded). (B) mRNA levels of the *jp* gene in *jp*\(^{+}\) wild type OrR flies, and in heterozygotes and homozygotes for the UAS-\(jp\) insertion do not show any significant differences. (C) Survival of the progeny expressing the *jp* RNAi driven by each one of the four *Gal4* drivers tested. The dotted line indicates the expected proportion of control flies expected (mendelian proportions 1:1 or 1:2) and the white portion of the bar the proportion observed. (D) Relative mRNA levels of *jp* in the OE and KD genotypes generated by crossing to two different *Gal4* lines compared to the control flies bearing
only the Gal4 construct. In bar diagrams data are represented as mean ± SEM. One-way Anova \(*p < 0.05, \quad ***p < 0.001\).
Figure 2. Muscular deficits in OE and KD flies. (A) Survival curves of the control (Mhc-Gal4), OE and KD flies; log-rank (Mantel-Cox) test shows significant differences with the control genotype. (B, B’) Neuromuscular competence of the three genotypes at 1 and 4 weeks of age estimated in the negative geotaxis assay (B, n=3 ≥15 flies each) and in the flight assay (B’, n=30). (C-C’) Semi-thin sections of the
thorax of control (C), OE (C’) and KD (C’’) flies at 4 weeks of age. (D) Area occupied by the IFMs in semi-thin sections at 1 and 4 weeks of age (n≥6, 3 sections analysed per individual). (E-E’’) TEM of ultra-thin longitudinal sections of the IFMs of control (E), OE (E’) and KD (E’’) flies at 4 weeks of age; under each panel the mitochondrial circularity index is indicated (n=3, 60 mitochondria per individual). (F) Relative mitochondrial genomic DNA; in each genotype and age the proportion of mitochondrial to nuclear genomic DNAs was calculated, and the results are displayed as mitochondrial genomic DNA abundance relative to the control genotype at each age. (G) Muscle fibre ultrastructure in a 1-week-old control fly, diads are indicated by white arrowheads, representing the normal morphology of these structures. The inset shows a wild type diad with indication of the sarcoplasmic reticulum (SR) and T-tubule (TT) components. (H) Examples of aberrant morphology such as elongated SR cisternae (EL) and diads embedded in the mitochondria (EM) in 1-week old OE fly muscles. (I) Abnormal rudimentary (RU), dysmorphic (DY) or vacuolated (VA) diads (arrowheads) in 1-week old KD fly muscles, usually next to disorganised mitochondrial cristae. Scale bars are 100 μm in C-C’’, 2 μm in E-E’’ and 1 μm in G-I. In bar diagrams data are represented as mean ± SEM, One-way Anova **p < 0.01, ***p < 0.001.
Figure 3. Cardiac dysfunction in OE and KD flies. (A) Survival curves of the control (GMH5-Gal4), OE and KD flies; log-rank (Mantel-Cox) test shows significant differences with the control genotype. (B-B’’) Cardiac function parameters altered in the experimental genotypes at 1 week of age (n≥15 per genotype): end systolic diameter (B), end diastolic diameter (B’) and fractional shortening (B’’). (C) Representative M-mode traces (vertical movement of the heart walls in 14 s) of semi-intact Drosophila hearts from the different genotypes; ESD are indicated in red and EDD are indicated in blue. (D-D’’) Semi thin sections of adult hearts of 1-week-old flies of the control (D), OE (D’) and KD (D’’) genotypes. Red bars represent heart wall thickness measurements like the ones that have been used for the quantitation in E. (E). Quantitation of heart wall thickness in the three genotypes as mean pixels (n≥10, 3 measurements per sample). (F-F’’) Phalloidin staining of dissected hearts with DAPI staining of nuclei reveals the normal myofibril structure in control 1-week-old flies (F) and abnormal morphologies in same
age OE (F’) and KD (D’’) hearts; white arrows point to gaps, areas devoid of myofibrils. (G) Quantitation of the proportion of surface with gaps in the myofibrillar staining of five hearts of different genotypes (n=5). Scale bars are 10 μm in D-D’’ and F-F’’. In bar diagrams data are represented as mean ± SEM. One-way Anova *p < 0.05, **p < 0.01, ***p < 0.001.
**Figure 4. Neuronal alterations in the OE and KD genotypes.**

A) Survival curves of the control (elav-Gal4), OE and KD flies; log-rank (Mantel-Cox) test shows significant differences with the control genotype. 

B) Bang sensitivity analyses of the three genotypes a 1 and 4 weeks of age, represented as recovery time after mechanical-stress-induced paralysis. (n=4, ≥10 flies per experiment).

(C-C’) SEM image of a wild type control eye (GMR-Gal4, C) and higher magnification showing the stereotypical hexagonal arrangement of the ommatidia and the inter-ommatidial bristles (C’). 

(D-D’) In the eye of a OE fly the eye has a rough aspect (D); and the ommatidia have lost the regular pattern and have supernumerary bristles (D’). 

(E-E’) KD fly eyes have a wild-type aspect (E) and structural arrangement...
(E’). (F-F’) The defects in UAS-jp eyes are corrected by co-expression of UAS-jpRNAi. (G) SEM of an ultra-thin section of a control fly ommatidium, showing the wild type trapezoidal arrangement with seven rhabdomeres. (H) Structure of an ommatidial section of an OE fly with extra rhabdomeres, as a result of the recruitment of extra photoreceptor neurons. (I) Loss of rhabdomeres, and hence photoreceptor neurons, in a KD ommatidial section. (J) Distribution of photoreceptor neuron number in ommatidia of the three genotypes at 1 week of age (n=4, ≥60 ommatidia per section). Scale bars are 100 μm in C-F, 20 μm in C’-F’ and 2 μm in G-I. In bar diagrams data are represented as mean ± SEM. One-way Anova *p < 0.05.
Figure 5. Jp levels modify the phenotype of pathological Htt and SCA3 poly-Q expansions. (A-A’’)

Progressive degeneration and de-pigmentation over 4 weeks upon expression of Htt-pQ93 in the eye
driven by GMR-Gal4. (B-B’’) Co-expression of Jp ameliorates the de-pigmentation. (C-C’’) Co-
expression of jp RNAi induces earlier faster progressing de-pigmentation. Neither GMR-Gal4 alone (D),
nor GMR-Gal4-driven UAS-jp (E) or UAS-jpRNAi (F) produce any de-pigmentation at 4 weeks. (G)
Expression of SCA-Q89 produces eye depigmentation which is evident at four weeks. (H) Co-expression of Jp reduces depigmentation. (I) Co-expression of jp RNAi increases depigmentation.
Figure 6. Notch-like phenotypes in OE flies. (A) Detail of the normal number and distribution of head vibrissae, notum microchaetae and sternopleural bristles in a control Act-Gal4/+ fly. (B) Bristles of these types are increased in number in flies of the Ac-Gal4, UAS-jp genotype (OE). (C) Duplicated scutellar macrochaetae in an OE fly. (D) Delta-shaped contacts of the wing veins with the wing margin in an OE fly. (E) Morphology of a wild-type control fly wing (rn-Gal4/+). (F) rn-Gal4, UAS-jp fly wing with expanded wing veins, delta-shaped contacts with the wing margin and decreased wing blade size. (G) rn-Gal4, UAS-jpRNAi wings have wild type morphology. (H) Quantitation of the blade area in wings of the genotypes shown in E-G shows that only OE wings have a significant difference with the control (n≥11). In bar diagrams data are represented as mean ± SEM. One way-Anova ***p < 0.001.
Figure 7. Levels of Jp expression modify the phenotype of a Dl mutant allele. (A-C) At 29ºC, wing blades of control nub-Gal4/+ (A), OE (B) and KD (C) genotypes have the same phenotypes as flies of the same genotypes cultured at the standard temperature. (D) Wing of a Dl6B37 fly displaying the typical Dl phenotype of engrossed veins and delta-shaped contacts with the wing margin. (E) over-expression of Jp in a Dl background enhances the phenotype. (F) Expression of the jp RNAi in a Dl background strongly suppresses the wing vein phenotypes. In each panel insets show a higher magnification of the area where measurements were performed, the white line indicates the length of the contact of vein L2 with the wing margin used in G. (G) Quantitation of the length of the contact of vein L2 with the wing margin in flies of the genotypes represented in A-F (n≥17 for each genotype). In bar diagrams data are represented as mean ± SEM. One-way Anova ***p < 0.001.
Figure S1. Strategy to generate the UAS-<i>jp</i> strain. Representation of the <i>P(XP)<i>jp<sup>do4563</sup> insertion before and after FLP-mediated removal of the upstream UAS promoter. Forward (F) and reverse (R) priming sites are indicated by green arrows and the expected amplicon sizes are indicated next to each insertion. The gel shows the bands obtained in PCR amplification with genomic DNA of Oregon<i>R</i> (negative control), and the two <i>P(XP)<i>jp<sup>do4563</sup> versions.
Figure S2. Metabolomic analysis of Mhc-Gal4 OE and KD fly thoraxes. (A, B) OPLS-DA models allow discrimination between the control and OE genotypes (A) and also between the control and KD genotypes (B). (C) Relative abundance of metabolites, estimated as integrated peak area in the NMR spectrogram, showing statistically significant differences compared to the control.
Figure S3. Unaffected cardiac parameters. Cardiac parameters that do not show any statistically significant differences with the control flies: heart period (HP), diastolic interval (DI), sistolic interval (SI) and arrhythmia index (AI), in parameters units are seconds.
**Figure S4. Analysis of neurodevelopmental vs neurodegenerative effects.** To minimise developmental defects and highlight neurodegeneration, flies were cultured at 18°C to tamper GMR-Gal4 expression during development, and then for a week at 25°C to get higher levels of Gal4 in differentiated retinal neurons. Retinas of control and KD flies were analysed at both time points, 1 day after eclosion and after 1 week at 25°C. (n=3, ≥60 ommatidia each)
Figure S5. Alteration of Jp levels with nub-Gal4. The area of wing blades from flies bearing the nub-Gal4 driver without any UAS construct (control), with UAS-Jp (OE), with UAS-JpRNAi (KD) or with both (OE + KD) was estimated. Only the OE wing blades have a statistical reduction in size, which is recovered by co-expression of UAS-JpRNAi (n≥18; *** p < 0.001).