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REVIEW

Fly for ALS: *Drosophila* modeling on the route to amyotrophic lateral sclerosis modifiers

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Abstract

Amyotrophic lateral sclerosis (ALS) is a rare, devastating disease, causing movement impairment, respiratory failure and ultimate death. A plethora of genetic, cellular and molecular mechanisms are involved in ALS signature, although the initiating causes and progressive pathological events are far from being understood. *Drosophila* research has produced seminal discoveries for more than a century and has been successfully used in the past 25 years to untangle the process of ALS pathogenesis, and recognize potential markers and novel strategies for therapeutic solutions. This review will provide an updated view of several ALS modifiers validated in C9ORF72, SOD1, FUS, TDP-43 and Ataxin-2 *Drosophila* models. We will discuss basic and preclinical findings, illustrating recent developments and novel breakthroughs, also depicting unsettled challenges and limitations in the *Drosophila*-ALS field. We intend to stimulate a renewed debate on *Drosophila* as a screening route to identify more successful disease modifiers and neuroprotective agents.

Keywords Experimental animal models · Genetic modifiers · Neuroprotection · Therapeutics

Introduction

The rare, devastating amyotrophic lateral sclerosis (ALS) or Lou Gehrig's disease causes movement impairment, respiratory failure and ultimate death. Epidemiology studies establish that most ALS cases (about 90%) are classified as sporadic ALS (sALS) with no clear genetic linkage [1], and that autosomal dominant (the most diffuse), autosomal recessive, and X-linked inherited forms of familial ALS (fALS) can all co-exist and show mutations in more than 20 different genes [2–4]. The relationship among the genetic, clinical phenotypes and pathological subtypes of ALS has become clearer in the past years, although the shared contribution of both genetic and environmental factors is not defined yet.

There is currently no cure for the disease, and treatments can only alleviate symptoms, prevent complications, possibly slow disease progression, and perhaps offer social and emotional support [5].

Despite much knowledge has been obtained up till now in dissecting the mechanisms of ALS [4, 6], scientists are still debating for instance about the pathogenic initiators versus the propagators of the disease, and if there is a primum movens, or simply the summation of multiple harmful events are causative of ALS. In this increasingly complex scenario, there is urgent need for genetic modifiers and efficacious therapeutics available to patients.

Drosophila responds well to this quest, thanks to its powerful genetic manipulation and forthright phenotypic impact. The reasons why the invertebrate *Drosophila melanogaster* has been successfully used in research for over a century are: (i) the fruit flies are easy to maintain if compared to animal models requiring controlled animal facilities; (ii) a vast progeny resource is obtained in a limited time, because the fruit fly breeds quickly and lays many eggs. In addition, *Drosophila* genome shares about 60% homology with the roughly 46,830 human protein- and regulatory RNA-coding genes [7]; nearly 50% of the fly protein sequences

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have mammalian homologs, and approximately 75% of the known human disease genes have a recognizable equivalent in *Drosophila* [8], thus enabling cross-investigations into genetic inheritance and pathology [9]. This adds to the enormous experimental advantages deriving from the marginal logistic and cost-effective requirements for *Drosophila* maintenance and, most importantly, from the avoidance of all ethical issues surrounding many animal models [10]. As such, *Drosophila* becomes a timely and relevant topic of discussion in the ALS field.

Experimental and clinical ALS findings

Classical ALS (Charcot type) is diagnosed after the recognition of both upper and lower motor neuron symptoms that occur concomitantly and with a worsening symptomatology, but that exclude other pathologies explaining the same symptoms. Muscle's weakness extended to all skeletal muscles and paralysis regionally spreading over time, are universally experienced in all patients, although the rate of ALS progression can be quite variable from one person to another [11]. Upper motor neuron symptoms include hyperreflexia, increased muscle tone and slowing of fast voluntary movements. Lower motor neuron signs comprise weakness, muscle wasting and fasciculation. In approximately 65% of patients, ALS initiates with weakness in limb muscles and consequent voluntary movement impairment (characterized by tripping, grasping difficulties, dropping things, abnormal fatigue, cramps and twitches); in the remaining 35%, with weakness in bulbar muscles and consequent disarticulation of speech, changes in vocal tone, swallowing difficulties [12]. Rarely, disease presentation includes weakness in respiratory or axial muscles [13]. In about 50% of affected individuals, the neurodegenerative course spreads to the frontal and anterior temporal lobes, causing a variable extent of executive dysfunctions, language impairments and humoral changes with uncontrollable periods of laughing or crying. Approximately 10% of patients develop frontotemporal dementia (FTD) by degeneration of cortical neurons in the frontal and anterior temporal lobes [14, 15]. Since ALS hits only motor neurons, the senses of sight, touch, hearing, taste, and smell are not affected, and for many people also, muscles of the eyes and bladder are often spared. The clinical manifestation of ALS is extremely variable in terms of site and age at onset, disease progression, proportion of upper versus lower motor neuron involvement, and rate of FTD incidence. Even in families with a monogenic cause of ALS, disease presentation is unpredictable, suggesting the existence of important disease-modifying factors [16, 17]. The adverse effects of ALS on respiratory muscles limit the survival to 2–5 years after disease onset, but many people can live 5, 10 or even more years.

In sALS and fALS, the initial onset is given by cellular and molecular changes that most likely precede the clinical onset. A motor neuron begins to degenerate when the accumulated molecular pathology exceeds a certain, still undefined, threshold. Then, symptoms are triggered by focal neuronal changes that can either occur in every motor neuron, or be limited to the affected motor neuron. Regional spread of symptoms is in turn caused by summation of degenerating neurons. ALS warning signs are characterized by damage to neuromuscular junction (NMJ), demyelination, axonal retraction, and loss of motor neuron cell bodies, in addition to the occurrence of astrogliosis, microgliosis and neuro-inflammation [18]. Ubiquitin-positive inclusions and enhanced axonal outgrowth and dendritic branching are instead observed in the surviving neurons [19]. The direct involvement of skeletal muscle is the subject of several studies, although its participation to the neuro-degenerative process is still discussed. There is compelling evidence proposing that ALS muscles suffer from oxidative stress, mitochondrial dysfunction, and bio-energetics disturbances, and the way by which different types of myofibers are affected might depend on their contractile and metabolic features [20]. Although the immune system components play a true role in early stages of ALS, more focused studies are required to determine how, when, and where the immune and inflammatory processes are crucial to disease progression [21–25].

Advantages and limitations of promoting ALS research through *Drosophila*

At present, *Drosophila* is extensively used as a genetic model for ALS [26, 27], providing valuable mechanistic information on disease insurgence and progression. What makes *Drosophila* an excellent ALS organism is the straightforward genetic manipulation, the multiple ways of drug delivery (by feeding, injection, inhaling), the different available modalities for testing disease progression (larval crawling and adult fly climbing tests, eclosion rate, lifespan assay, phenotypical observations, electrophysiological screenings) and, most of all, the quite faithful correspondence between what observed in ALS patients and what reproduced in flies in terms of survival, motor disabilities, motor neuron degeneration, presence of cellular inclusions, mitochondrial abnormalities and oxidative damage [28] (Fig. 1). The steps for studying ALS in *Drosophila* are to generate pathogenic models through available genetic techniques [29], to monitor disease progression, to screen putative disease modifiers and therapeutic compounds. Remarkably, *Drosophila* can further allow a precise and spatio-temporally controlled expression of human transgenes, thanks to fine-tuned transgenesis programs, such as the Gal4-Uprstream Activating Sequence

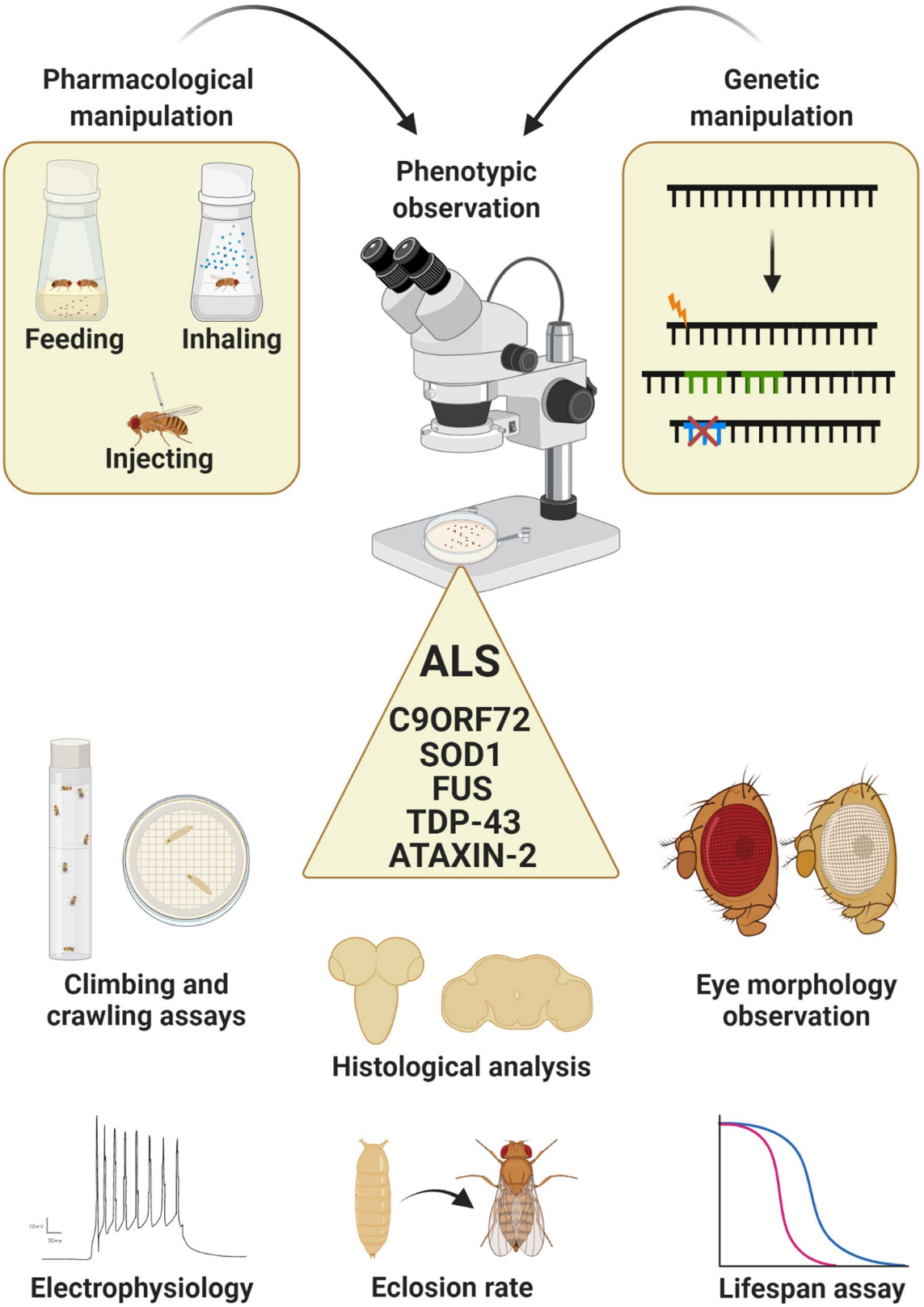

Fig. 1 *Drosophila* as a magnifying tool for dissecting ALS features. An important advantage of fly modeling is the possibility to evaluate the effectiveness of pharmacological treatments and genetic manipulation through different experimental read-outs

(Gal4-UAS) [30], the temporal and regional gene expression targeting (TARGET), or the gene-switch systems [31] (Fig. 2).

Of note, the *Drosophila* visual system assumes a great relevance in fruit fly modeling, because about two-thirds of the vital genes in the *Drosophila* genome are involved in the eye development [32]. This makes the fly's eye a powerful genetic tool to study patterning, growth, survival, neurodevelopment, neuro-degeneration and, not least, complex diseases comprising ALS. The compound eye of *Drosophila* consists of approximately 800 units (ommatidia), each containing eight photoreceptor neurons (R1-R8), two primary pigment cells and four lens cells. Ommatidia are separated from one another by a slight septum composed of

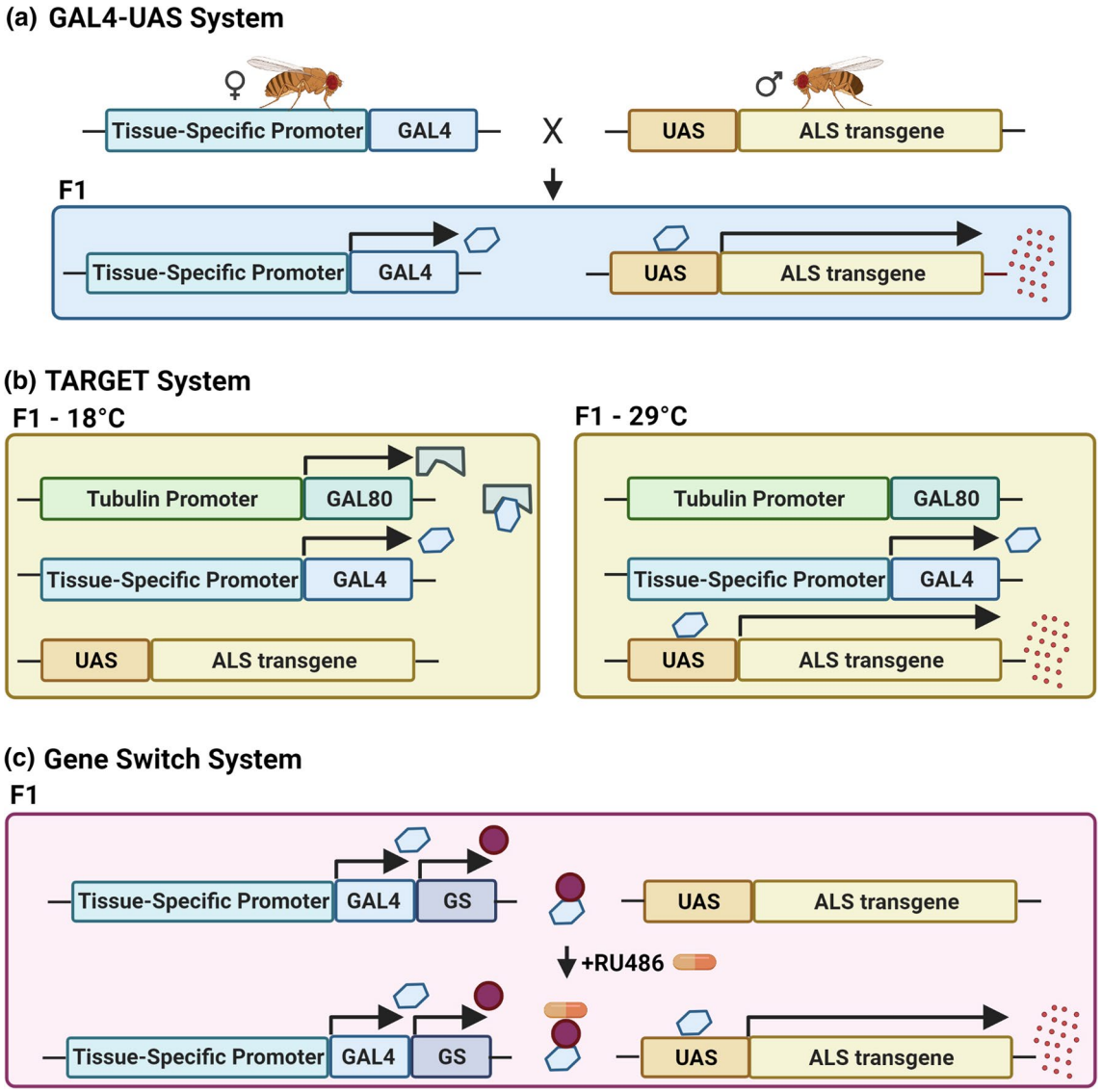

Fig. 2 Graphical scheme of the available genetic systems to ectopically express human transgenes in *Drosophila*. *Drosophila* modeling allows spreading the expression of a transgene exclusively within a specific tissue, thanks to different transgenesis programs. **a** The bipartite Gal4-UAS system is characterized by the use of two lines of flies: the first one carrying the yeast transcription factor GAL4 under the control of a tissue-specific (or stadium-specific) promoter, and the second one carrying the transgene downstream the UAS domain (the GAL4 binding site). By crossing these two lines, a progeny (F1) expressing the transgenic protein only in the tissues where the GAL4 is active will be generated. **b** The temporal and regional gene expression targeting (TARGET) system permits a fine-tuned temporal control of the expression, in a more precise manner than Gal4/UAS. The ubiquitous tubulin promoter spreads the expression of a temperature-sensitive Gal80 that, at 18 °C, binds the Gal4 and blocks the transgene transcription. Shifting the temperature at 29 °C determines the Gal4 deliverance and its interaction with UAS sequences, thus allowing the transgene production. **c** The Gene Switch system is characterized by a hormone-inducible Gal4: feeding flies with a nutritional medium supplemented with RU486 (mifepristone) determines the Gal4 activation and consequently the initiation of the transcriptional process

six secondary pigment cells, three tertiary pigment cells, and three bristle complexes [33, 34]. Any subtle insult modifying the geometry of this precisely ordered structure, leads to a visible aberrant morphological phenotype, such as smaller or larger eyes, changes in ommatidia, changes in bristles, and loss of pigmentation. Consequently, the introduction of a putative genetic modifier in an altered eye genetic background may suppress or enhance the disease eye phenotype, and therefore provide interesting evidence for the involvement of a new player in the pathogenic scenario.

Despite these several advantages, we must be aware that some limitations about *Drosophila* modeling exist too. The anatomy of the brain and other major organs within the fruit fly are quite different from that of humans, and there is lack of adaptive immune system. Other issues can be related to behavioral studies, because in-depth cognitive abilities are absent in the fly. Not least, some drug effects are sometimes reported to be different in the fly compared to human, suggesting that future directions for preclinical drug development must also consider comparative analysis in different animal models. However, we believe that *Drosophila* remains an invaluable organism for performing rapid and cost-effective screening particularly of genetic modifiers and putative neuroprotective agents, and for dissecting the genetics and mechanistic pathways of ALS. The search for novel diagnostic tools and breakthrough therapeutics will surely rely on *Drosophila* to empower effective solutions for ALS patients.

ALS disease modifiers identified in *Drosophila* models

Considering that the main pathological traits of ALS may be easily recapitulated in *Drosophila*, several transgenic fly models have been successfully generated [27, 35] and used to screen putative genetic modifiers and molecules with a potential neuroprotective action [36]. In the next sections, we will present a detailed updated view of different ALS modifiers, able to suppress the neurodegenerative phenotype induced by the expression in *Drosophila* of *C9ORF72*, *SOD1*, *FUS*, *TARDBP* or *Ataxin-2* transgenes, together representing about 70% of all fALS cases.

C9ORF72-ALS modifiers

Chromosome 9 Open Reading Frame 72 (*C9ORF72*) gene, the most frequent fALS causative gene [37, 38], is composed of 12 exons, two of which are non-coding. C9ORF72 protein has been recently described as an autophagy regulator and component of guanine nucleotide exchange factor complex [39, 40]. Within the *C9ORF72* first intron, the hexa-nucleotide GGGGCC (G_4C_2) may be repeated from 2 to 23 times in wild-type gene [41], but its aberrant expansion reaching hundreds or thousands of repeats, has been found in ALS and FTD patients [42–44]. A consistent group of evidence shows that higher is the number of repeats above the 23-threshold, more severe is the phenotypical alteration [45–51].

The proposed and non-mutually exclusive mechanisms underlying C9ORF72-ALS pathogenesis are [52]: (i) RNA-mediated toxicity by direct sequestration of RNA-binding proteins (RBP); (ii) production through Repeat-Associated Non-ATG (RAN) translation of toxic dipeptide repeats (DPRs) that accumulate in the cytoplasm and become cytotoxic [53–56]; (iii) loss-of-function mechanisms determining a decrease of C9ORF72 protein [57, 58]. By considering that the *Drosophila* genome has no ortholog for *C9ORF72* [59], the loss-of-function effect cannot be studied in the fly.

Concerning the RNA toxicity aspect, it is well known that expanded and GC-rich transcripts are prone to form secondary structures, such as R loops and G-quadruplexes that normally antagonize the RNA Polymerase II transcription process [60]. Recent papers highlighted the role of DRB-sensitivity-inducing factor (DSIF) and of polymerase-associated factor 1 (PAF1C) complexes. The authors showed that both facilitate the transcription machinery and promote the expression of G_4C_2 repeats, by resolving RNA secondary structures [48, 51, 61]. In particular, the downregulation of a core component of DSIF complex [62] partially rescues eye degeneration and increases lifespan in a fly model expressing 49 repeats of G_4C_2 hexa-nucleotide [48]. Likewise, reduced expression of *Drosophila* PAF1C components modulates toxicity at various levels: C9ORF72-flies carrying RNAi construct for PAF1C subunits show extended lifespan, better climbing performance, rescued eye phenotype and reduced presence of brain vacuoles [51]. Still focusing on the RNA toxic role, other results showed that targeted overexpression in fly eyes or motor neurons (by tissue-specific GAL4 drivers) of the RBP Purα rescues the neurodegenerative phenotype [45]. Consistently, Celona and collaborators report that the overexpression of the RBP Zfp106, suppresses hexa-nucleotide repeat expansion (HRE)-induced neurotoxicity in a *Drosophila* C9ORF72-ALS model expressing a $(G_4C_2)_{30}$ construct in glutamatergic neurons [63]. Recently, also the ALS-associated RBP Matrin-3 was described as an in vivo modulator of C9ORF72-ALS pathogenesis; its overexpression, indeed, is able to rescue eye neurodegeneration, lifespan and motor performance in a fly model carrying a (G_4C_2) expansion [64]. Remarkably, removing the RNA-recognition-motif (RRM) domain from Matrin-3 nullifies the neuro-protective effect. Taken together, these results confirm that modulating the transcriptional process of RNA, or overexpressing its trapped interactors, may become a promising target to treat C9ORF72-ALS. In a recent paper, Jiao and co-workers reported that the enzyme Topoisomerase 2 (Top2) may be considered as a newcomer in the ALS modifier field.

They showed that pharmacologically reducing Top2 expression ameliorates G_4C_2-induced neurotoxicity, in a fly model of C9ORF72-ALS [65], thus unveiling another potential ALS therapeutic target.

As known, G_4C_2 HRE may be transcribed in both sense and anti-sense directions and then translated in five toxic DPRs: poly-GA (glycine–alanine), poly-GR (glycine–arginine), poly-PR (proline–arginine), poly-PA (proline–alanine), and poly-GP (glycine–proline) [55, 56, 66]. By exploiting the *Drosophila* eye as screening tool, Lee and colleagues, performed an extended in vivo RNAi analysis and identified 80 suppressors and 27 enhancers of C9ORF72-induced toxicity [49], thus providing a strong evidence of DPR involvement in C9ORF72-ALS pathogenesis. Although their involvement in RAN translation mechanism is still to be clearly defined, canonical translation factors eIF4B and eIF4H were identified as modifiers of DPR-induced toxicity: their downregulation leads to a reduced production of toxic peptides in a C9ORF72-ALS fly model [67].

Several recent reports showed that DPR-induced toxicity is limited to arginine-rich DPRs [46, 47, 49, 68–71]; e.g., Mizielinska and collaborators showed that only poly-$(GR)_{36}$ and poly-$(PR)_{36}$ constructs, individually expressed in the *Drosophila* eye or motor neurons, lead to neurodegeneration, but not poly-$(GA)_{36}$ and poly-$(PA)_{36}$ [46]. Likewise, Freibaum and co-workers reported that poly-$(GP)_{47}$ and poly-$(GA)_{50}$ do not contribute to any degenerative phenotype, when ectopically expressed in flies [47]. In contrast, some recent papers highlighted that *Drosophila* "short repeats models" could not be totally informative about the disease mechanism, considering that ALS/FTD patients' expansion is often greater than 500 repeats [72–74]. On this context, West and co-workers generated a *Drosophila* model carrying more than 1000 toxic repeats. They showed that each of the five DPRs has its unique pathological profile and contributes to neurodegeneration in a specific way. Authors revealed, moreover, that co-expressing specific DPRs determines new phenotypes not detected when pathogenic constructs are expressed one by one [75].

Arginine-rich DPRs were reported to impair Notch signaling and cause cytoplasmic aggregates [69]. Their expression in *Drosophila* glutamatergic neurons causes neurodegeneration and excitotoxicity with increased intracellular calcium and extracellular glutamate levels in the brain [76]. The inhibition of NMDA receptors in DPR-expressing glutamatergic neurons extends lifespan and rescues motor defects in *Drosophila* [76]. Considering that riluzole, one of the two approved drugs for ALS treatment [77], is an anti-glutamatergic agent, this study strongly supports the arginine-rich-DPRs/glutamatergic axis and its therapeutic value for C9ORF72-ALS treatment.

An additional cellular process increasingly involved in the C9ORF72-ALS scenario is the nucleocytoplasmic transport (NCT): consistent reports indicate the possibility of modifying C9ORF72-induced toxicity by modulating the expression of proteins belonging to this trafficking pathway. Of note, through a large-scale deficiency screening in a *Drosophila* model expressing a $(G_4C_2)_{58}$ transgene in the eye, Freibaum and colleagues found that many components of NCT suppress or enhance eye ommatidia deregulation [47]. Likewise, through a RNAi screening in a C9ORF72 *Drosophila* model expressing a construct with 25 PRs in the eye, Boeynaems and colleagues found that modulation of importins, exportins and other nuclear pore components, improves C9ORF72-ALS-induced altered eye phenotype [78]. Consistently, *Drosophila RanGAP* (human *RanGAP1* ortholog) overexpression, or pharmacological treatment with the nuclear export inhibitor KPT-276, rescues the neurodegeneration in a $(G_4C_2)_{30}$ fly model [79]. The same group recently proposed that NCT disruption triggers autophagy dysfunction, leading to chronic protein stress and neuronal death. C9ORF72-mediated neurodegeneration is rescued by nuclear import of the autophagy-factor Mitf/TFEB [80]. Furthermore, increased cytosolic calcium levels have been recently reported to be crucial to regulate TDP-43 NCT and reduce its aggregation in a C9ORF72-fly model, introducing the calcium-Calpain A-Importin α3 axis as a new potential therapeutic target [81]. Interesting insights came recently from Lee and colleagues about the molecular chaperone Sigma-1 receptor, whose mutations have been already linked to fALS [82–85]. They demonstrated that Sigma-1 receptor presence at nuclear pore (it co-localizes with endogenous RanGAP and nucleoporins Nup62 and RanBP2) counterbalances G_4C_2-HRE toxic effect and that its overexpression rescues eye defects, aberrant motor behavior and electrophysiological deficits in a *Drosophila* $(G_4C_2)_{30}$ ALS model [86].

The HRE- or DPR-mediated NCT disruption unavoidably causes an abnormal reallocation of several proteins within the nuclear and cytoplasmic compartments; recently, Ortega and collaborators identified many proteins that in C9ORF72-ALS scenario are shifted in the cytosolic fraction. Among these, the translation termination and nonsense-mediated decay (NMD) regulator eRF1 resulted to have the strongest neuroprotective effect in a $(G_4C_2)_{36}$ fly model: the overexpression of its ortholog ETF1 rescues eye depigmentation and reduces poly-GR DPR levels [87]. This finding strengthens the NMD pathway as a potential ALS therapeutic target, as also confirmed by a recent group of evidence reporting UPF1 (the master regulator of NMD) as a strong in vivo modulator of C9ORF72-induced neurotoxicity [87–90].

Remarkably, many of the above-described results (Table 1) were validated for the first time in a fly model, confirming *Drosophila* as an ideal tool to dissect molecular mechanisms, identify putative genetic modifiers, and assess

Table 1 Disease modifiers identified in *Drosophila* C9ORF72-ALS models

Disease modifier	Fly transgenic construct	Phenotypic rescue	Refs.
DSIF complex downregulation	GMR-Gal4 > UAS(G_4C_2)$_{49}$;Stp4$_{RNAi}$	Eye morphology, lifespan	[48]
PAF1C complex downregulation	GMR-Gal4 > UAS(G_4C_2)$_{49}$;dPaf1$_{RNAi}$ Elav-Gal4 > UAS(G_4C_2)$_{49}$;dPaf1$_{RNAi}$	Brain vacuoles, eye morphology, motor behavior, lifespan	[51]
Purα overexpression	GMR-Gal4 > UAS-(G_4C_2)$_{30}$;UAS-Purα	Eye morphology	[45]
Zfp106 overxpression	Oak-Gal4 > (G_4C_2)$_{30}$;UAS-Zfp106	Motor behavior, NMJ	[63]
Matrin-3 overexpression	GMR-Gal4 > UAS-(G_4C_2)$_{30}$;UAS-Matr3 GMR-Gal4 > UAS-(G_4C_2)$_{36}$;UAS-Matr3 GMR-Gal4 > UAS-(G_4C_2)$_{58}$;UAS-Matr3	Eye morphology	[64]
	ElavGS-Gal4 > UAS-(G_4C_2)$_{30}$;UAS-Matr3	Motor behavior, lifespan	
Topoisomerase 2 downregulation	GMR-Gal4 > UAS-(G_4C_2)$_{30}$;Top2$_{RNAI}$	Eye morphology	[65]
Teniposide	GMR-Gal4 > UAS-(G_4C_2)$_{30}$ OK371-Gal4 > UAS-(G_4C_2)$_{30}$	Motor behavior	
Genistein	GMR-Gal4 > UAS-(G_4C_2)$_{30}$ OK371-Gal4 > UAS-(G_4C_2)$_{30}$	Motor behavior	
eIF4B downregulation	GMR-Gal4 > LDS(G_4C_2)$_{EXP}$;UAS-eIF4B$_{RNAi}$	Eye morphology	[67]
eiF4H downregulation	GMR-Gal4 > LDS(G_4C_2)$_{EXP}$;UAS-eIF4H$_{RNAi}$	Eye morphology	
vGlut downregulation	D42-Gal4 > UAS-GR$_{36}$;vGLUT$_{RNAi}$	Motor behavior, lifespan	[76]
Nuclear components modulation	GMR-Gal4 > (PR)$_{25}$	Eye morphology	[78]
KPT-276 treatment	GMR-Gal4 > UAS(G_4C_2)$_{30}$	Eye morphology	[79]
RanGap overexpression	GMR-Gal4 > UAS(G_4C_2)$_{30}$;UAS-RanGap	Eye morphology	
Mitf genomic duplication	GMR-Gal4 > UAS-30R;Mitf(Dp) ElavGS > UAS-30R;Mitf(Dp) vGlut-Gal4 > UAS-30R;Mitf(Dp)	Eye morphology, eclosion rate, motor behavior, autophagolysosomal pathway	[80]
Increased cytosolic calcium concentration	ppk1a-Gal4 > UAS-SERCA$_{RNAi}$; UAS-Imp a3$_{RNAi}$;UAS-TBPH-Flag-HA	TBPH aggregates	[81]
Sigma-1 receptor overexpression	GMR-Gal4 > UAS(G_4C_2)$_{30}$;UAS-Sig-1R Elav-Gal4 > UAS(G_4C_2)$_{30}$;UAS-Sig-1R	Eye morphology, motor behavior	[86]
eRF1/ETF1 overexpression	GMR-Gal4 > UAS-(G_4C_2)$_{36}$;ETF1^{OE}	Eye morphology, Poly-GR levels	[87]
UPF1 overexpression	GMR-Gal4 > UAS-(G_4C_2)$_{30}$;UAS-UPF1 GMR-Gal4 > UAS-(G_4C_2)$_{36}$;UAS-UPF1	Eye morphology	
UPF1 overexpression	D42-Gal4 > UAS-GR$_{36}$;UAS-UPF1	Lifespan, motor behavior	[88]
Tranilast	Elav-Gal4 > UAS-GR$_{36}$ Elav-Gal4 > UAS-PR$_{36}$	Lifespan, motor behavior	[88]
UPF1 overexpression	GMR-Gal4 > UAS-(G_4C_2)$_{30}$;UAS-UPF1	Eye morphology	[90]
Phospholipase D pathway modulation	GMR-Gal4 > UAS(G_4C_2)$_{30}$	Eye morphology	[91]

the contribution of different cellular patterns in a multifaceted disease like ALS.

SOD1-ALS modifiers

Superoxide dismutase 1 (SOD1) is an evolutionarily

conserved ubiquitous protein catalyzing dismutation of superoxide into hydrogen peroxide and dioxygen. In 1993, *SOD1* was identified as the first gene whose mutations were linked to ALS [92, 93]; to date, more than 200 different point mutations of the *SOD1* gene have been related both to fALS and sALS [94, 95]. These mutations cause deregulation of cellular pathways by combination of loss and gain of toxic functions [96–99], all leading to the damage of motor neurons as main crucial feature. One of the most prominent pathological mechanisms of SOD1-related ALS is an above-threshold presence of oxidative stress caused by unprocessed free radicals and high production of reactive oxygen/nitrogen species [100].

During the last twenty-five years, different *Drosophila* transgenic models have been developed for studying pathogenic mechanisms linked to human *SOD1* gene mutations [101–103], all sharing reduced climbing abilities, increased SOD1 protein aggregation and mitochondrial dysfunctions in motor neurons [102, 103].

Drosophila carrying the human *SOD1* transgene with the missense mutation G85R (SOD1-G85R) [102], highlighted the potential neuroprotective effect of several different antioxidant molecules (Table 2). In detail, De Rose and collaborators demonstrated that adult flies expressing the SOD1-G85R transgene in motor neurons show higher survival rate and better motor performance if extracts of *Withania somnifera* (a plant with antioxidant, anti-inflammatory properties) are added to standard nutritional medium [104]. Interestingly, *W. somnifera* is protective in a loss-of-function TDP-43 fly model too, determining a partial rescue of climbing and walking activity [105]. Moreover, urate treatment enhances survival, attenuates motor impairments, reduces oxidative damage and increases antioxidant defense in human SOD1-G85R *Drosophila* [106]. Interestingly, the antioxidant α-lipoic acid exerts neuroprotection in ALS flies expressing SOD1-G85R in motor neurons, by extending survival rate, rescuing motor impairment, activating the ERK/Akt pathway and indirectly regulating the expression of antioxidant enzymes [107].

The natural antioxidant fisetin extends lifespan, improves climbing activity and activates the ERK pathway in SOD1 mutant flies. Fisetin-treated flies have less SOD1 aggregates in brain respect to untreated flies, and the hypothesis was formulated that fisetin may regulate autophagy in ALS pathogenesis [108].

Recently, Zhang and collaborators showed that γ-oryzanol ameliorates ALS symptomatology in SOD1-G85R flies by reducing oxidative stress and free radicals damage, and sustaining lifespan and motor abilities [109].

FUS-ALS modifiers

Fused-in-Sarcoma/Translocated-in-Liposarcoma (FUS/TLS) is a multi-functional DNA/RNA-binding protein found in RNA-containing stress granules (SG) [110–112], regulating gene expression [113], RNA metabolism [114–116] and splicing [117, 118]. Only recently, *FUS* was described as a bicistronic gene encoding for an alternative peptide (alt-FUS), whose suppression is neuroprotective in a *Drosophila* FUS-overexpressing model [119].

ALS-linked *FUS* mutations were identified in 2009 [120, 121] and over the years, different FUS-related *Drosophila* models have been generated [122–124], all sharing ALS pathogenic hallmarks. Ectopic expression of wild-type or mutant FUS, triggered by a tissue-specific Gal4 driver, has been reported to impact on fly motor behavior, lifespan, ommatidial morphology and eclosion rate [122, 125–130]. Consistently, different mutations in *Drosophila FUS* ortholog, *cabeza* (*caz*), determine reduction of lifespan, locomotor abnormalities and reduced eclosion rate [126, 131, 132]; these defects are rescued by introducing a wild-type human FUS in the mutated background [126], thus highlighting the high level of conservation between *caz* and *FUS* genes.

To date, many suppressors of FUS-induced toxicity have been identified in fly models (Table 3). For instance, overexpression of *ter94*, fly ortholog of *Valosin-containing protein* (VCP) gene and ALS-causing itself [133], rescues motor neuron defects in a *caz*-knockdown background, whilst *ter94*-inactivation exacerbates the neurodegenerative phenotype [134]. Interestingly, further evidence of the interaction between FUS and VCP has been recently reported in a human iPSC cellular line obtained from VCP-mutant motor neurons, where FUS mis-localization was found [135].

Recently, Kankel and colleagues performed some independent modifier screening experiments using two different fly models expressing the fALS causing mutant transgenes

Table 2 Disease modifiers identified in *Drosophila* SOD1-ALS models

Disease modifier	Fly transgenic construct	Phenotypic rescue	Refs.
Withania somnifera extracts	D42-Gal4 > hSOD1^{G85R}	Motor behavior, lifespan	[104]
Urate	D42-Gal4 > hSOD1^{G85R}	Motor behavior, oxidative stress, lifespan	[106]
α-lipoic acid	D42-Gal4 > hSOD1^{G85R}	Motor behavior, lifespan	[107]
Fisetin	D42-Gal4 > hSOD1^{G85R}	Motor behavior, SOD1 aggregates	[108]
γ-oryzanol	D42-Gal4 > hSOD1^{G85R}	Motor behavior, oxidative stress, lifespan	[109]

Table 3 Disease modifiers identified in *Drosophila* FUS-ALS models

Disease modifier	Fly transgenic construct	Phenotypic rescue	Refs.
altFUS suppression	Elav-GS > UAS-altFUS^{Ø}	Motor behavior	[119]
ter-94 overexpression	GMR-Gal4 > UAS-Caz-IR$_{363\text{-}399}$;UAS-ter94	Motor behavior, lifespan	[134]
Phospholipase D pathway modulation	GMR-Gal4 > UAS-FUS^{R521C}	Eye morphology	[91]
Drosophila muscleblind downregulation	GMR-Gal4 > FUS;mbl$_{RNAi}$ D42-Gal4 > FUS;mbl$_{RNAi}$	Eye morphology, motor behavior, NMJ	[136]
Drosophila Rm62 overexpression	GMR-Gal4 > UAS-FUS; Rm62^{OE} GMR-Gal4 > UAS-FUS^{R521C}; Rm62^{OE} GMR-Gal4 > UAS-FUS^{R518K}; Rm62^{OE}	Eye morphology	[143]
	ElavGS-Gal4 > UAS-FUS; Rm62^{OE} ElavGS-Gal4 > UAS-FUS^{R521C}; Rm62^{OE} ElavGS-Gal4 > UAS-FUS^{R518K}; Rm62^{OE}	Motor behavior	
Nucleoporin 154 downregulation	CCAP-Gal4 > UAS-FUS;Nup154$_{RNAi}$	FUS-induced neurotoxicity	[129]
Exportin 1 downregulation	CCAP-Gal4 > UAS-FUS;Xpo1$_{RNAi}$	FUS-induced neurotoxicity	
Nucleoporin 62 downregulation	D42-Gal4 > UAS-FUS^{R521C}; Nup62$_{RNAi}$ D42-Gal4 > UAS-FUS^{R518K}; Nup62$_{RNAi}$ ElavGS-Gal4 > UAS-FUS^{P525L}; Nup62$_{RNAi}$	Nuclear abnormalities, lifespan	[145]
Hippo downregulation	GMR-Gal4 > UAS-Caz-IR;hpo^{KS240} Elav-Gal4 > UAS-Caz-IR;hpo^{KS240}	Eye morphology, motor behavior	[147]
Hippo downregulation	GMR-Gal4 > UAS-FUS;hpo GMR-Gal4 > UAS-FUS;jun	Eye morphology	[148]
Parkin overexpression	GMR-Gal4 > UAS-FUS;UAS-parkin Elav-Gal4 > UAS-FUS;UAS-parkin	Eye morphology, motor behavior	[149]

FUS (missense mutation R521C) and TDP-43 (missense mutation M337V). By analyzing the eye phenotype of flies co-expressing the putative modifier together with the fALS transgenes, they identified a complex array of ALS phenotype enhancers and suppressors, many of which affecting both FUS- and TDP-43-expressing strains. Interestingly, the strongest genetic modifiers of both FUS and TDP-43 toxicity were tested on a third ALS model, expressing a $(G_4C_2)_{30}$ construct. A cohort of genes with effects on diverse ALS models were found, opening the possibility to identify relevant genes or pathways shared by different ALS forms [91]. Of note, the Authors identified the phospholipase D pathway as one of the major modifiers of ALS phenotypes, by validating its positive effects not only in multiple fly models, but also in SOD1-G93A mice [91]. Another unbiased genetic screening highlighted *muscleblind Drosophila* gene as a modifier of FUS-induced toxicity: its functional inactivation in a mutant FUS background rescues ommatidia defects, improves motor abilities and recovers NMJ defects [136]. Of note, *muscleblind* human ortholog, *MNBL1* gene, affecting RNA trafficking, splicing and processing has been previously linked to several neurodegenerative disease [137–140]. Very recently, through a RNA-sequencing approach, Fortuna and co-workers identified the RNA helicase DDX17 (DEAD-Box Helicase 17), whose activity is necessary for transcription and splicing processes [141, 142], as a new modulator of FUS-induced toxicity. In particular, the overexpression of *Rm62*, the *DDX17 Drosophila* ortholog, ameliorates eye degeneration and climbing performances in wild-type and mutant FUS expressing flies [143]. Additionally, authors unveiled the role of DDX17 in DNA damage response pathway, thus presenting DSB repair as a new potential therapeutic target for FUS-induced ALS treatment.

As for C9ORF72-ALS, mounting evidence suggests a pivotal role for NCT in FUS-ALS too [144]. Downregulation of Nucleoporin 62, Nucleoporin 154 and of Exportin 1 (key modulators of nuclear export) indeed reduces FUS-induced toxicity in a FUS-overexpressing fly model [129, 145].

A recently discovered *caz* modulator is *Hippo*, the fly ortholog of Mammalian Sterile 20-like kinase 1, whose pathway is involved in tumor suppression [146]. *Caz* downregulation-induced defects in motor neurons are suppressed by introducing loss-of-function mutations of *Hippo* [147]. Similarly, flies expressing a wild-type or mutant human FUS transgene in the eye show a rescue of neurodegeneration if components of *Hippo* or c-Jun N-terminal Kinase (JNK) signaling pathways are modulated [148], thus suggesting

these pathways as new potential therapeutic targets for FUS-ALS treatment.

Not least, a recent result shows that the E3 ubiquitin ligase Parkin exerts a neuroprotective effect in a *Drosophila* model overexpressing FUS in muscle tissues: it is peculiar that Parkin expression does not directly modulate FUS protein levels, but rescues the pathological phenotype recovering mitochondrial defects caused by FUS proteinopathy [149].

TDP-43-ALS modifiers

TAR-binding protein 43 (TDP-43) is a 43 KDa RBP involved in mRNA stability [150], miRNA processing [115, 151] and splicing regulation [152]. It has been linked to ALS because it was found as a core component of neuronal inclusion bodies in ALS patients [153–155]. Over the years, 48 different point mutations in the *TARDBP* gene encoding for TDP-43 have been identified as ALS-causing [156]. Contextually, many *Drosophila* models have been generated. Targeted overexpression of wild-type or mutant human TDP-43 causes reduced lifespan, eclosion failure, impaired motor functions, axon swelling and cytoplasmic toxic aggregates [157–163]. Similar phenotypes are obtained by overexpressing *TAR DNA binding homolog* (*TBPH)* gene, *TARDBP Drosophila* ortholog, which indeed determines climbing defects, cytoplasmic accumulations and eclosion failure [164, 165]. Moreover, loss of *TBPH* causes defective motor behaviors and eclosion defects, abnormalities at NMJ and reduced lifespan: these symptoms are rescued by human TDP-43 expression [164, 166], suggesting not only the high evolutionary conservation of TDP-43 [167], but also that any positive or negative deviation from a threshold expression of *TBPH* might induce ALS-like features in *Drosophila*. Interestingly, Romano and collaborators showed that *TBPH* depletion either in neurons or glia impairs the organization of glutamate GluRIIA receptors at the NMJ, supporting the hypothesis of not-only-neuronal origin of TDP-43-ALS [168–170].

It is not surprising that FUS and TDP-43 induce neurodegeneration through co-incidental processes, and the effects of their overexpression in *Drosophila* are similar, given their functional similarity. Different studies demonstrated not only their interaction [125, 171, 172], but also TDP-43 capability to act as FUS-induced toxicity enhancer in flies [125, 126].

In the last decade, different TDP-43 fly models have been used to perform compound or genetic modifier screening (Table 4). Autophagy upregulation may be a useful therapeutic approach [173] and evidence showed that autophagy reactivation through rapamycin reduces toxic aggregation rate, improves lifespan and partially rescues motor impairments in a *Drosophila* model overexpressing *TBPH* in motor neurons [174]. Interestingly, also *TBPH*-deficient flies show better motor abilities and higher survival rate by powering up autophagy through overexpression of *Autophagy Related 7* gene [175].

Mitochondrial fragmentation is a prominent common feature of ALS [176–178]: flies pan-neuronally expressing TDP-43 show extremely small or fragmented mitochondria [179]. *Mitofusin* gene is a key regulator of mitochondrial fusion process and its mRNA and protein levels are reduced by TDP-43 overexpression: a reduced rate of fusion may determine mitochondrial fragmentation. Importantly, restoring *mitofusin* expression ameliorates spontaneous walking and climbing of wild-type or mutant TDP-43-expressing flies [179]. Recently, Sun and colleagues demonstrated that both Parkin and PINK1, master regulators of mitophagy, are deregulated by TDP-43 overexpression. Furthermore, they showed that upregulation of Parkin and downregulation of PINK1 delay climbing defects and extend lifespan in a fly model of TDP-43-ALS [180]. Taken together, these data suggest that mitochondrial dynamics, for many aspects, cover an important role in TDP-43-ALS pathogenesis and could become valuable target for therapeutic compounds.

Drosophila TBPH regulates the expression of several genes encoding for pre-synaptic terminal proteins influencing synaptic transmission, such as Futsch, Syntaxin 1A and Synapsin [166, 169]. In a *Drosophila*-ALS model expressing TDP-43 in motor neurons, a reduction of both *futsch* mRNA and protein was registered at the NMJ [181]. Remarkably, *futsch* ectopic expression rescues motor impairment, reduces TDP-43 aggregates, extends lifespan and recovers NMJ abnormalities [181]. An additional therapeutic target is suggested by a recent study on a *Drosophila* model of TDP-43 loss-of-function: *cacophony* is a gene directly regulated by TBPH [182] that encodes for a voltage-gated calcium channel, whose mRNA reduction is linked to TBPH loss. Restoring Cacophony levels in all neurons, or specifically in motor neurons, in a TBPH-/-background, leads to the rescue of motor disturbances caused by *TBPH* loss-of-function [183].

The interaction between TDP-43/TBPH and its RNA targets is crucial to determine the pathogenic effect of proteinopathy: interestingly, recent evidence demonstrates that removing TDP-43 RNA-recognition-motif (RRM) domains leads to reduction of toxic effects. Ihara and colleagues, for instance, have generated a fly ALS model expressing TDP-43 in retinal neurons, characterized by photoreceptor vacuolar degeneration, and thinning of the retina. ALS-induced altered eye phenotype is completely rescued by preventing the binding ability of TDP-43 through mutations or deletion in its RRM domains [184]. Likewise, a pharmacological approach produced the same result, thanks to in silico docking and biochemical assays, the compound 6-(3-[4-fluorobenzyl]-3-(hydroxymethyl)piperidin-1-yl) pyrazine-2-carboxamide was identified to reduce the TDP-43 ability to bind disease-linked nucleic acids and ameliorate

Table 4 Disease modifiers identified in *Drosophila* TDP-43-ALS models

Disease modifier	Fly transgenic construct	Phenotypic rescue	Refs.
Phospholipase D pathway modulation	GMR-Gal4 > UAS-TDP-43^{M337V}	Eye morphology	[91]
Withania somnifera extracts	D42-Gal4 > UAS-TBPH$_{RNAi}$	Motor behavior, lifespan	[105]
Rapamycin	D42-Gal4 > UAS-TBPH	Motor behavior, lifespan	[174]
ATG7 overexpression	Act-Gal4 > TBPHΔ23/Δ23;UAS-Atg7	Motor behavior, lifespan	[175]
Mitofusin overexpression	Elav-Gal4 > UAS-TDP-43;UAS-Marf	Motor behavior	[179]
Parkin overexpression	Elav-Gal4 > UAS-TDP-43;UAS-Parkin	Motor behavior, lifespan	[180]
PINK-1 downregulation	Elav-Gal4 > UAS-TDP-43;PINK1$_{RNAi}$	Motor behavior, lifespan	
Futsch overexpression	D42-Gal4 > UAS-TDP-43;P(EP)futsch^{EP1419}	Motor behavior, NMJ, lifespan	[181]
Cacophony overexpression	Elav-Gal4 > UAS-Cacophony;TBPH^{DD96} D42-Gal4 > UAS-Cacophony;TBPH^{DD96}	Motor behavior	[183]
6-(3-(4-fluorobenzyl)-3-(hydroxymethyl) piperidin-1-yl)pyrazine-2-carboxamide	D42-Gal4 > UAS-TDP-43	Motor behavior	[185]
Drosophila REF1 downregulation	GMR-Gal4 > UAS-TDP-43; UAS-ATXN2-32Q;UAS-Ref1$_{RNAi}$	Eye morphology	[186]
Medium-chain fatty acids	D42-Gal4 > UAS-TDP-43	Motor behavior	[190]
Palmitoyltransferase downregulation	D42-Gal4 > UAS-TDP-43;Cpt1$_{RNAi}$ D42-Gal4 > UAS-TDP-43;Cpt2$_{RNAi}$	Motor behavior	
Glycolysis upregulation	D42-Gal4 > UAS-TDP-43;UAS-Glut3 D42-Gal4 > UAS-TDP-43;UAS-PFK	Motor behavior	[191]
Drosophila Atx2 downregulation	GMR-Gal4 > dAtx2^{X1} Elav-Gal4 > dAtx2^{X1}	Eye morphology, lifespan	[197]
PABP2 downregulation	GMR-Gal4 > UAS-TDP-43;PABP2^{LOF} GMR-Gal4 > UAS-TDP-43 ^{D169G};PABP2^{LOF} GMR-Gal4 > UAS-TDP-43 ^{A315T};PABP2^{LOF}	Eye morphology (worsening)	[201]
	D42-Gal4 > UAS-TDP-43;PABP2^{LOF} D42-Gal4 > UAS-TDP-43 ^{D169G};PABP2^{LOF} D42-Gal4 > UAS-TDP-43 ^{A315T};PABP2^{LOF}	Motor behavior (worsening)	
ATXN2 PAM2 domain depletion	GMR-Gal4 > UAS-TDP-43;UAS-ATXN2-32Q^{ΔPAM2} ElavGS-Gal4 > UAS-TDP-43;UAS-ATXN2-32Q^{ΔPAM2}	Eye morphology, motor behavior, lifespan	[199]
Poly-ADP-ribose polymerase downregulation	GMR-Gal4 > UAS-TDP-43;PARP$_{RNAi}$ Elav-Gal4 > UAS-TDP-43;PARP$_{RNAi}$	Eye morphology, motor behavior, lifespan	[203]
Tankyrase downregulation	GMR-Gal4 > UAS-TDP-43;TkrsIR Elav-Gal4 > UAS-TDP-43;TkrsIR	Eye morphology, lifespan	[202]

motor capabilities in flies overexpressing wild-type or mutant TDP-43 in motor neurons [185]. Interestingly, also the downregulation of RNA export process was reported to be neuroprotective in multiple fly ALS models: for instance, the functional inactivation of *Drosophila REF1*, fly ortholog of human ALYREF mRNA exporting factor, is able to mitigate TDP-43, TDP-43/ATXN2-32Q and G_4C_2 neurotoxicity [186]. Specifically, *REF1* knockdown suppresses fly eye neurodegeneration, reduces TDP-43 mRNA and protein levels, and G_4C_2 mRNA and poly-GA rates in their respective fly models [186].

Together with motor neuron damage, ALS patients exhibit bioenergetics deficits and hyper-metabolism [187–189]. A metabolomics study on wild-type and mutant TDP-43 overexpressing larvae presenting the common ALS phenotypical hallmarks, showed significant alteration in lipid metabolism and deficit in carnitine shuttle responsible for long-chain fatty acid import into the mitochondria, lipid beta-oxidation and ATP production [190]. Interestingly, feeding these flies with medium-chain fatty acids not needing the carnitine shuttle to reach the mitochondrial matrix, improves larval motor abilities. Moreover, downregulating the expression

of the carnitine shuttle major components palmitoyltransferase 1 or 2, suppresses motor impairment [190]. The same group moreover proved that TDP-43 expressing flies show alterations in glucose metabolism. Interestingly, they demonstrated that glycolysis upregulation rescues TDP-43 proteinopathy, through overexpression of GLUT-3 glucose transporter or phosphofructokinase in motor neurons [191]. Taken together, these data provide an important link between TDP-43-induced proteinopathy and metabolic processes, highlighting a further potential therapeutic target for ALS treatment.

Further insights about TDP-43-ALS are described in the next paragraph, where some aspects of TDP-43 and Ataxin-2 interaction are also elucidated.

ATAXIN-2 as a TDP-43 modifier

Human *Ataxin-2* gene (*ATXN2*) is the causative gene of Spinocerebellar Ataxia Type 2 (SCA2), because of a pathologic CAG repeat expansion (more than 34, respect to 22 in normal alleles) in its first exon, causing an abnormal poly-Q tract in ATXN2 protein [192, 193]. ATXN2 is involved in RNA stability, degradation and translation, and is crucial for SG assembly [194, 195]. Interestingly, through studies on *Drosophila*, an intermediate trinucleotide expansion (from 27 to 33 repeats [196]) has been demonstrated to be ALS-associated, confirming *ATXN2* as an ALS susceptibility gene [197].

Atx2 gene, *ATXN2 Drosophila* ortholog, is essential for fly viability and is involved in translation control and RNP assembly [198]. Its loss causes bristle and eye defects together with motor impairments, whereas the effects of its overexpression range from locomotor deficits to lethality [194]. In a TDP-43 overexpressing ALS fly model, *atx2* overexpression exacerbates ALS phenotype, further reducing lifespan and worsening eye ommatidia degeneration; conversely, *atx2* functional inactivation extends lifespan and rescues eye aberrant morphology [197]. Moreover, transgenic flies carrying human *ATXN2* gene with 32 CAGs show enhanced toxicity in a TDP-43-ALS background, with lower survival rate and reduced climbing performance [199]. Through PAM2 domain, human ATXN2 binds PABP protein, key regulator of SG formation [200] and whose reduction exacerbates TDP-43-induced toxicity in different ALS models [201]. Strikingly, flies expressing a domain-mutant *ATXN2-32CAGs* transgene encoding for ATXN2 protein without PAM2 domain, no longer modify TDP-43 toxicity pattern, indicating that PABP and SG formation may have a pivotal role in TDP-43-induced neurodegeneration [199].

In this regard, recent evidence suggests that poly-ADP-ribosylation (PARylation) plays a crucial role in regulating TDP-43 and SG dynamics: the reduction of PARylation levels suppresses SG formation and TDP-43 recruitment to SGs [202, 203]. Genetic and pharmacological inhibition of poly-ADP-ribose polymerase (PARP) rescues eye neurodegeneration, extends lifespan, and ameliorates motor performance in a TDP-43-overexpressing fly model [203]. Similarly, downregulation of poly-ADP-ribosyltransferase Tankyrase, involved in TDP-43 SG inclusion, suppresses the eye altered phenotype and fly lifespan reduction [202]. These results provide strong indications that modulation of PARylation may unveil therapeutic strategies not explored yet for ALS treatment. Further insights about RNA granules indicate that deletion of *Atx2* intrinsically disordered regions domain, affecting RNP granule formation, is sufficient to rescue the neurodegenerative phenotypes of both FUS-related and C9ORF72-related fly ALS models [198], strongly suggesting that regulation of RNP granule assembly may represent an important strategy to counterbalance neurodegeneration in a wide spectrum of ALS-associated pathogenic contexts.

Conclusion

As we have detailed, *Drosophila* genetics has played an important role in discovering the involvement of several cellular and molecular pathways in ALS pathogenesis, and identifying potential disease modifiers. Although it may seem a paradox, great similarities in basic and network biochemistry occur and are conserved in all animals; this has allowed *Drosophila* being extensively studied as a powerful ALS model in which to discern components of pathways, understand disease mechanisms, investigate responses to genetic modifiers, and finally identify new therapeutics. Indeed, some exciting advances obtained through *Drosophila* have already facilitated our understanding of how input information is integrated and translated into an output motor response, and of how impaired molecular signaling and neuronal circuits that coordinate motor behavior can detrimentally impact on ALS pathology. Although a wealth of information has been collected so far, numerous mechanistic, basic research and clinical questions still remain unanswered. In particular, the master challenge in *Drosophila*-ALS research is making new genetic modifiers and therapeutic molecules safely and more rapidly available to patients, while the master limitation is of course the jump from flies to humans. Despite this insurmountable paradigm shift, genetic manipulations in *Drosophila* offer higher efficiency in targeting ALS disease genes than in higher organisms and provide greater proficiency in both dissecting pathological responses and screening therapeutic compounds.

We fairly anticipate and believe that *Drosophila* overcoming its model borders may now renew its scientific consent

in ALS, and we prospect a time when research on ALS will keep sturdily investing on *Drosophila*.

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Declarations

Conflict of interest The authors declare that they have no competing interests.

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