**Using *Drosophila melanogaster* to discover human disease genes: An educational primer for use with “Amyotrophic Lateral Sclerosis modifiers in *Drosophila* reveal the Phospholipase D pathway as a potential therapeutic target”**

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***Answers to the guided reading questions***

To conduct genome-wide screens for ALS modifier genes, the authors collected females from transgenic *Drosophila* strains expressing the ALS-associated *hFUSR521C* or *hTDP-43M337V* alleles, each controlled by a UAS regulatory element (Figure 1 of (Kankel *et al.* 2020)). Each strain also carries the *GMR-GAL4* driver, which produces GAL4 protein in cells of the eye, thereby causing expression of the human ALS-associated proteins there. These females were mated to males from the Exelixis collection, each strain of which carries an individual insertion mutation*.* The researchers selected the F1 progeny lacking the balancer chromosomes, based on their non-Curly wings and their non-Tubby bodies, as shown in the Punnett squares below. Note that animals homozygous for either balancer do not survive to adulthood.

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| --- | --- | --- | --- | --- |
|  | ***GMR-GAL4;*** ***UAS-hFUSR521C*** | ***GMR-GAL4; TM6B*** | ***CyO;*** ***UAS-hFUSR521C*** | ***CyO; TM6B*** |
| ***Exelixis insertion mutation*** | *GMR-GAL4; UAS- hFUSR521C/ insertion* | *GMR-GAL4; TM6B/ insertion* | *CyO; UAS-hFUSR521C/ insertion* | *CyO; TM6B/ insertion* |
| ***Balancer*** ***(CyO or TM6B)*** | *GMR-GAL4; UAS- hFUSR521C/ Balancer* | *GMR-GAL4; TM6B/ Balancer* | *CyO; UAS-hFUSR521C/ Balancer* | *CyO; TM6B/ Balancer* |

|  |  |  |
| --- | --- | --- |
|  | ***GMR-GAL4,*** ***UAS-hTDP-43M337V*** | ***CyO*** |
| ***Exelixis insertion mutation*** | *GMR-GAL4, UAS- hTDP-43M337V/ insertion* | *CyO/ insertion* |
| ***Balancer (e.g., CyO)*** | *GMR-GAL4; UAS- hTDP-43M337V/ CyO* | *CyO/ CyO* |

A normal adult *Drosophila* eye has a highly organized structure of facets and a red color (Figure 1B, taken from a control flycarrying only the *GMR-GAL4* driver). In contrast, eyes from flies carrying *GMR-GAL4*; *UAS-hFUSR521C* (*GMR-hFUSR521C*) and *GMR-GAL4*, *UAS-hTDP-43M337V* (*GMR-hTDP-43M337V*) appear rough and disorganized, with loss of the normal pigmentation and ordered lens structure, indicating that expression of human ALS alleles in the fly eye induces degeneration (Figure 1C and 1H, respectively). To demonstrate that the modifier screen strategy could work, eyes from flies carrying a heterozygous Exelixis insertion mutation in an endogenous *Drosophila* gene along with either *GMR-hFUSR521C* (Figure 1D-F) or *GMR-hTDP-43M337V* (Figure 1I-K) were examined. The test genes *Hsc70Cb* and *dco* are fly orthologs of human genes implicated in ALS-related phenotypes, and *dSETX* is the fly ortholog of another human ALS gene. The degeneration in these eyes varies compared to flies expressing the ALS-associated human allelesalone, with the *dSETX* and *Hsc70Cb* mutations suppressing the degenerative eye phenotype of both *GMR-hFUSR521C* and *GMR-hTDP-43M337V*, and the *dco* mutation suppressing the eye phenotype of *GMR-hFUSR521C* but enhancing the phenotype of *GMR-hTDP-43M337V*. These proof of principle experiments demonstrate that the degenerative eye phenotype caused by expression of human ALS alleles can be modified by heterozygous Exelixis insertions in *Drosophila* genomic loci. The authors also found that reduced dosage of the gene *Ask1*, previously linked to ALS-related phenotypes, can suppress the eye phenotype of both *GMR-hFUSR521C* and *GMR-hTDP-43M337V* (Figure 1G and 1L, respectively). Instead of using an Exelixis mutation, they knocked down *Ask1* by RNAi.

After demonstrating that the degenerative eye phenotype caused by expression of human ALS-associated alleles could be dominantly modified, the authors screened 15,500 Exelixis lines for both ALS alleles, thus requiring 31,000 crosses. Crosses were examined 15 days after initiation, making the F1 flies about 1-5 days old, due to the approximately 10-day development period of *Drosophila melanogaster* from egg to adult. The screens recovered 637 insertions that modify *GMR-hFUSR521C* and 553 insertions that modify *GMR-hTDP-43M337V* (Figure 2). Of these, 432 hits modify both, yielding a total of 758 unique hits, which represent 5% of the total screened loci. This percentage is similar to the percentage of hits in other screens conducted using the Exelixis collection (e.g., see (Kankel *et al.* 2007; Sen *et al.* 2013). Although 432 hits affect both *hFUSR521C* and *hTDP-43M337V* transgenes, the common hits do not always affect the two transgenes in the same way, i.e., some hits are enhancers for one transgene but suppressors for the other and vice versa. For example, *sasd07239* suppresses *GMR-hFUSR521C* but enhances *hTDP-43M337V*,while*pumd04225* and *orbd06989* enhance *GMR-hFUSR521C* but suppress *hTDP-43M337V*. This may suggest that the two human ALS alleles converge on a common molecular pathway but affect its activity in opposite ways.

Following the original screens, the authors retested their hits using another common ALS-associated allele, caused by expansion of the GGGGCC hexanucleotide repeat in the *c9orf72* gene (Figure 3). As with *hFUS* and *hTDP-43* alleles, previous studies had shown that expression in the fly eye leads to cytotoxicity and degeneration (Xu *et al.* 2013). The degenerative phenotype of *GMR-c9orf72(G4C2)30* flies, which manifests as black necrotic patches in the eye, worsens progressively with age. As before, the authors screened for genes that can dominantly modify the phenotype, but rather than retesting all 15,500 Exelixis insertions, the authors chose 84 strong suppressors that were common to both original screens. They examined eye degeneration over time in flies carrying *GMR-c9orf72(G4C2)30* along with each Exelixis insertion mutation or, in some cases, RNAi knockdown. This experiment improves on the previous strategy by providing a quantitative read-out of the degeneration phenotype and by following progressive degeneration over time, which better simulates the degenerative nature of ALS. The results are exemplified by five strong suppressors, each of which lowers the penetrance of eye degeneration caused by *GMR-c9orf72(G4C2)30* at the ages of 1, 3, and 6 weeks old (Figure 3I). Penetrance of degeneration was calculated at each time point by the number of flies showing necrosis in the eye divided by the total number of flies examined. Despite the advantages of quantifying in this manner, this experiment is more labor intensive and takes longer than the original method, making it less practical for a genome-wide screen. Of 84 loci tested, 56 (67%) suppress the *GMR-c9orf72(G4C2)30* phenotype and 10 enhance it, thus identifying 66 loci that modify all three ALS alleles (79% of those tested). Thus, most of the strongest suppressors identified in the original screens appear to be common modifiers of many ALS pathways.

In the original screens, the transgenes carry human alleles, but the authors next turned their attention to the *Drosophila* ortholog *dTDP-43* (Figure 4). They examined a mutant variant of the fly gene that is equivalent to an ALS-associated human allele (*dTDP-43N493D*) in comparison to wild-type *dTDP-43* (*dTDP-43WT*). Previous studies had found that expression of *hTDP-43* mutated in its nuclear localization signal (NLS) causes severe eye degeneration in flies, so Kankel et al. also examined a transgenic strain carrying *dTDP-43* with an NLS mutation (*dTDP-43mNLS*)(Ritson *et al.* 2010; Miguel *et al.* 2011). As with the human transgenes, expression of *dTDP-43WT* and *dTDP-43mNLS* causes eye degeneration, although this property cannot be ascertained for *dTDP-43N493D* due to larval lethality. Unlike the human *TDP-43M337V* transgene, all three *Drosophila* transgenic proteins form cytoplasmic aggregates in cells of the larval eye imaginal disc, which is the larval precursor of the adult eye, and in larval motor neurons to varying extents (Figure 4D-O). Surprisingly, this aggregation property is visibly modified by only seven hits out of the 16 tested, even though all 16 loci can suppress eye degeneration. As TDP-43 aggregation is strongly implicated in ALS pathogenesis, this observation raises important questions about its centrality to disease progression and may open up new lines of investigation.

In human ALS patients, it is the motor neurons specifically that degenerate, whereas the photoreceptors of the fly eye are sensory neurons. Therefore, it was important to validate the results of the screens by expressing ALS-associated alleles in motor neurons with the *OK371-GAL4* driver*.* The anatomical site of contact between motor neurons and muscle tissue, called the neuromuscular junction (NMJ), is particularly disease-relevant (see (Cappello and Francolini 2017) for a detailed discussion of the NMJ in ALS), and *OK371-dTDP-43N493D* flies have severe NMJ defects, which are correlated with larval lethality (Figure 5A, E, H). Thus, the *Drosophila* cognate of an ALS-associated *TDP-43* allele is detrimental to motor neurons.

The genes *SF2,* *lilli,* and *klp98A* were chosen for validation at the NMJ because they were among the strongest common suppressors of the ALS photoreceptor degeneration models. As expected, mutations in *SF2* or *lilli* suppress the NMJ defect of *OK371-dTDP-43N493D*, suggesting that these genes are involved in neurotoxicity in ALS (Figure 5F, G, H). Surprisingly, *klp98A* shows no effect in the NMJ model, implying that its activity is limited to cell types other than motor neurons. This result highlights the importance of validating screen hits in motor neurons directly in order to assess their disease relevance.

From their screens, the authors home in on the Phospholipase D (PLD) pathway (schematized in Figure 9). The hypothesis that PLD is important for ALS progression is supported by five pieces of evidence, presented in Figure 6: (a) RNAi knockdown of *Drosophila PLD (dPLD)* suppresses the eye degeneration of *GMR-hTDP-43M337V* (Figure 6A-B)*,* (b) RNAi knockdown of *dPLD* suppresses the eye degeneration of *GMR-hFUSR521C* (Figure 6C-D)*,* (c) RNAi knockdown of *dPLD* suppresses the eye degeneration of *GMR-c9orf72(G4C2)30* (Figure 6E)*,* (d) RNAi knockdown of *dPLD* suppresses the NMJ defect of *OK371-dTDP-43N493D*(Figure 6I)*,* and (e) *dPLD* overexpression in motor neurons disrupts the NMJ (Figure 6H-I). By extension, other factors in the PLD pathway also would be expected to affect ALS phenotypes. For example, because RalA has been implicated as an activator of PLD, interfering with RaIA would be expected to reduce PLD activity and therefore behave similarly to PLD knockdown with respect to the ALS phenotypes.

As predicted, reducing *RalA* expression by RNAi knockdown or inhibiting its function with a dominant negative suppresses the degenerative phenotypes of the *GMR-hTDP-43M337V, GMR-hFUSR521C,* and *GMR-c9orf72(G4C2)30* fly models (Figure 7C-D, G-I). Moreover, reducing expression of another pathway component, Rgl, also suppresses ALS phenotypes (Figure 7B, F, J-M). These results demonstrate that the effects are not limited to PLD itself, with multiple molecular components in the same pathway capable of modifying the ALS phenotypes. Because downregulating PLD pathway components suppresses ALS phenotypes, we can surmise that ALS-associated allelesrequire active PLD to cause cytotoxicity. This suggests that PLD likely is downstream in the pathway, although this model requires confirmation by more detailed genetic and biochemical experiments.

Despite all of its advantages as an experimental model organism, elaborated above, the substantial evolutionary distance between *Drosophila* and humans raises the question of whether discoveries made in flies are directly applicable to medicine. For this reason, mice, which are more closely related to humans genetically, anatomically, and physiologically, are an important intermediate step in translating findings from basic research to human therapeutics. In wild-type mice, hindlimb and forelimb grip strength increases by ~10-30% between days 100 and 120, while inverted grip strength does not change (Figure 8D-H, blue bars). In contrast, ALS model mice that express the disease allele SOD1G93A display decreases in hindlimb, forelimb, and inverted grip strength that are improved modestly by mutations in *PLD1, PLD2*, or both (Figure 8C-H). These results are consistent with the observations in *Drosophila* that reducing PLD activity improves ALS phenotypes, and they nicely demonstrate how complementary data from multiple model systems are used to build a strong scientific argument.

In conclusion, Kankel et al. present a forward genetic screening strategy to identify loci involved in ALS using the powerful experimental tools and resources of *Drosophila melanogaster*. They show that this strategy can be used with a variety of ALS-associated alleles (including human disease alleles *hFUSR521C*, *hTDP-43M337V*, and *c9orf72(G4C2)30* and alleles of *Drosophila dTDP-43*) and in a variety of cellular contexts (including the adult fly eye, larval eye imaginal disc, and larval NMJ). Their data identify the PLD pathway as an important conserved factor for the disease even when initiated by different pathogenic proteins. Downregulation of PLD pathway components suppresses phenotypes caused by expression of ALS-associated alleles, which is consistent with patient data suggesting that upregulation of PLD pathway components accelerates disease onset. Together, the evidence from *Drosophila*, mice, and humans implicates the PLD pathway in disease progression and raises it as a potential avenue for further investigation to both understand and treat ALS. Because the genetic circuitry of ALS is highly complex and because the effects of PLD downregulation in the mouse ALS model are modest, targeting PLD may be most effective in combination with other therapies. The hope is that this work will synergize with other studies to illuminate successful treatment options for this devastating disease.

***References***

Cappello, V., and M. Francolini, 2017 Neuromuscular Junction Dismantling in Amyotrophic Lateral Sclerosis. Int J Mol Sci 18.

Kankel, M. W., G. D. Hurlbut, G. Upadhyay, V. Yajnik, B. Yedvobnick *et al.*, 2007 Investigating the genetic circuitry of mastermind in Drosophila, a notch signal effector. Genetics 177**:** 2493-2505.

Kankel, M. W., A. Sen, L. Lu, M. Theodorou, D. N. Dimlich *et al.*, 2020 Amyotrophic Lateral Sclerosis Modifiers in. Genetics.

Miguel, L., T. Frébourg, D. Campion and M. Lecourtois, 2011 Both cytoplasmic and nuclear accumulations of the protein are neurotoxic in Drosophila models of TDP-43 proteinopathies. Neurobiol Dis 41**:** 398-406.

Ritson, G. P., S. K. Custer, B. D. Freibaum, J. B. Guinto, D. Geffel *et al.*, 2010 TDP-43 mediates degeneration in a novel Drosophila model of disease caused by mutations in VCP/p97. J Neurosci 30**:** 7729-7739.

Sen, A., D. N. Dimlich, K. G. Guruharsha, M. W. Kankel, K. Hori *et al.*, 2013 Genetic circuitry of Survival motor neuron, the gene underlying spinal muscular atrophy. Proc Natl Acad Sci U S A 110**:** E2371-2380.

Xu, Z., M. Poidevin, X. Li, Y. Li, L. Shu *et al.*, 2013 Expanded GGGGCC repeat RNA associated with amyotrophic lateral sclerosis and frontotemporal dementia causes neurodegeneration. Proc Natl Acad Sci U S A 110**:** 7778-7783.