Title:

In vivo functional study of disease-associated rare human variants using *Drosophila*

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Keywords:

Human genetics and genomics, Mendelian diseases, rare and undiagnosed diseases, Undiagnosed Diseases Network (UDN), *Drosophila melanogaster*, variant of unknown significance (VUS), gene of uncertain significance (GUS), functional genomics, transgenic flies, UAS/GAL4 system, T2A-GAL4, electroretinogram (ERG)

Summary:

The goal of this protocol is to outline a process to design and perform *in vivo* experiments in *Drosophila melanogaster* to assess the functional consequences of rare variants that are associated with human diseases.

Abstract:

Advances in sequencing technology have made whole-genome and whole-exome datasets more easily accessible for both clinical diagnosis and cutting-edge human genetic research. Although a number of *in silico* algorithms have been developed to predict the pathogenicity of variants identified in these datasets, functional studies are critical to determine if specific genomic variants may affect protein function, especially for missense variants. In the Undiagnosed Disease Network (UDN) and other rare disease research consortiums, model organisms (MO) including *Drosophila*, *C. elegans*, zebrafish, and mice are actively being used to assess the function of putative human disease-causing variants. Here, we describe a protocol for functional assessment of rare variants that is being used in the UDN Model Organisms Screening Center *Drosophila* Core. The workflow begins with gathering human and MO information from multiple public databases using the MARRVEL tool to assess whether the variant is likely to contribute to patients' conditions and design effective experiments based on available knowledge and resources. Next, we generate genetic tools (T2A-GAL4 lines and UAS-human cDNAs) to assess the function of variants of interest in *Drosophila*. Upon development of these reagents, we perform a two-pronged functional assay based on rescue and over-expression experiments. In the rescue branch, we attempt to "humanize" the endogenous fly gene by replacing the orthologous *Drosophila* gene with reference or variant human transgenes. In the over-expression branch, we exogenously drive the reference and variant human proteins in a variety of tissues. In both cases, any scorable phenotype (e.g. lethality, eye morphology, electroretinogram) can be used as outputs irrespective of the disease of interest. Differences observed between reference and variant alleles suggest functional differences caused by the missense variant, thus suggesting pathogenicity. This protocol allows rapid *in vivo* assessments of putative human disease-causing variants for genes of both known and unknown function.

Introduction:

Patients with rare diseases often undergo an arduous journey referred to as the 'diagnostic odyssey' to obtain an accurate diagnosis[1]. Most rare diseases are thought to have a strong genetic origin, making genetic and genomics analyses critical elements of the clinical workup. In addition to candidate gene panel sequencing and copy number variation analysis based on chromosomal microarrays, whole-exome (WES) and whole-genome sequencing (WGS) technologies have become increasingly valuable tools over the past decade[2,3]. Currently, the diagnostic rate of identifying a known pathogenic variant in WES and WGS is ~25% (higher for pediatrics cases)[4,5]. For most cases that remain undiagnosed after receiving clinical WES/WGS, the issue is that there are too many candidate genes and variants. Next generation sequencing often identifies novel or ultra-rare variants in many genes in an individual's exome or genome, and interpreting whether these variants may contribute to disease phenotypes is challenging. For example, although most nonsense or frameshift mutations in genes are thought to be loss-of-function alleles due to nonsense mediated decay of the encoded transcript, truncating mutations found in the last exons escape this process and may function as benign or gain-of-function alleles[6]. Moreover, predicting the effect of a missense allele is a daunting task since it can result in a number of different genetic scenarios as first described by Herman Muller in the 1930s; amorph, hypomorph, hypermorph, antimorph, neomorph, or isomorph[7]. Numerous *in silico* programs and methodologies have been developed to predict the pathogenicity of missense variants based on evolutionary conservation, type of amino acid changes, position within a functional domain, allele frequency in the general population, and other parameters[8]. However, these programs are not a comprehensive solution to solving the complicated problem of variant interpretation. Interestingly, a recent study demonstrated that five broadly used variant pathogenicity prediction algorithms [Polyphen (genetics.bwh.harvard.edu/pph2)[9], SIFT (sift.bii.a-star.edu.sg)[10], CADD (cadd.gs.washington.edu)[11], PROVEAN (provean.jcvi.org/index.php)[12], and Mutation Taster (www.mutationtaster.org)] agree on pathogenicity ~80% of the time[8]. However, even when all algorithms agree, they return an incorrect prediction of pathogenicity up to 11% of the time. This not only leads to flawed clinical interpretation, but may also dissuade researchers from following up on new variants by falsely listing them as benign. One way to complement the current limitation of *in silico* modeling is to provide experimental data that demonstrates the effect of variant function *in vitro*, *ex vivo* (e.g. cultured cells, organoids), or *in vivo*.

In vivo functional studies of rare disease associated variants in MO have unique strengths[13], and have been adopted by many rare disease research initiatives around the world including the Undiagnosed Diseases Network (UDN) in the United States (undiagnosed.hms.harvard.edu) and the Rare Diseases Models & Mechanisms (RDMM) Networks in Canada (www.rare-diseases-catalyst-network.ca), Japan (irudbeyond.nig.ac.jp), Europe (solve-rd.eu) and Australia (www.functionalgenomics.org.au)[14]. In addition to these coordinated efforts to integrate MO

researchers into the workflow of rare disease diagnosis and mechanistic studies at a national scale, a number of individual collaborative studies between clinical and MO researches have led to discovery and characterization of new human disease-causing genes and variants[82-84]. In the UDN, a centralized Model Organisms Screening Center (MOSC) receives submissions of candidate genes and variants with a description of the patients' condition and assesses whether the variant is likely to be pathogenic using informatics tools and *in vivo* experiments. In Phase I (2015-2018) of the UDN, the MOSC comprised of a *Drosophila* Core [Baylor College of Medicine (BCM)] and a Zebrafish Core (University of Oregon) that worked collaboratively to assess cases. Using informatics analysis and a number of different experimental strategies in *Drosophila* and zebrafish, the MOSC has so far contributed to the diagnosis of 132 patients, the identification of 31 new syndromes[55], the discovery of several new human disease genes (e.g. *EBF3*[15], *ATP5F1D*[16], *TBX2*[17], *IRF2BPL*[18], *COG4*[19], *WDR37*[20]) and phenotypic expansion of known disease genes (e.g. *CACNA1A*[21], *ACOX1*[22]). In addition to projects within the UDN, MOSC *Drosophila* Core researchers have contributed to new disease gene discoveries in collaboration with the Center for Mendelian Genomics and other initiatives (e.g. *ANKLE2*[23], *TM2D3*[24], *NRD1*[25], *OGDHL*[25], *ATAD3A*[26], *ARIH1*[27], *MARK3*[28], *DNMBP*[29]) using the same set of informatics and genetic strategies that were developed for the UDN. Given the significance of MO studies on rare disease diagnosis, the MOSC was expanded to include a *C. elegans* Core and an additional Zebrafish core (both at Washington University at St Louis) for the Phase II (2018-2022) of the UDN.

In this manuscript, we describe an *in vivo* functional study **Protocol** that is actively being used in the UDN MOSC *Drosophila* Core to determine if missense variants have a functional consequence on the protein of interest using transgenic flies that express human proteins. The goal of this **Protocol** is to help MO researchers to work collaboratively with the clinical research groups to provide experimental evidence that the variant of interest has functional consequences and facilitate the clinical diagnosis. This **Protocol** will be most useful in a scenario in which a *Drosophila* researcher is approached by a clinical investigator who has a rare disease patient with a specific candidate variant in a gene of interest. This **Protocol** can be broken down into three elements; (1) Gathering information to assess the likelihood of the variant of interest being responsible for the patient phenotype and the feasibility of a functional study in *Drosophila*, (2) Gathering existing genetic tools and establishing new ones, and (3) Performing functional studies *in vivo*. The third element can further be subdivided into two sub-elements based on how one can assess the function of a variant of interest (rescue experiment or over-expression based strategies). It is important to note that this **Protocol** can be adapted and optimized to many scenarios outside of rare monogenic disease research (e.g. common diseases, gene-environment interaction, pharmacological and genetic screens to identify therapeutic targets). The ability to determine the functionality and pathogenicity of variants will not only benefit the patient of interest via providing an accurate molecular diagnosis but will also have broader impacts on translational and basic scientific research.

Protocol:

1. Gather human and MO information to assess the likelihood of a variant of interest being responsible for disease phenotypes and the feasibility of functional studies in *Drosophila*

1.1 Perform extensive database and literature searches to determine whether the specific genes and variants of interest are good candidates to explain the phenotype of the patient of interest. Some key questions that should be explored include:

Q1) Has this gene been previously implicated in other genetic disorders (phenotypic expansion of known disease gene) or is this an entirely new disease candidate gene [gene of uncertain significance (GUS)]?
Q2) At what frequency have the alleles of interest been seen in disease or control population databases?
Q3) Are there copy number variations that include this gene in disease or control population databases?
Q4) What are the orthologous genes in different MO species (e.g. mouse, zebrafish, *Drosophila*, *C. elegans*, yeast) and what are known about their functions and expression patterns?
Q5) Is the variant in a functional domain of the protein and is the amino acid of interest evolutionarily conserved?

Note: Answers to these five questions (**A1-5**) can be obtained by accessing a number of human and MO databases individually. Alternatively, one can quickly obtain a summary of these results using the MARRVEL (Model organism Aggregated Resources for Rare Variant ExpLoration, http://marrvel.org/) tool[30], which is described in-depth in an accompanying JoVE article[31]. See "Representative Results" section for specific examples. Additional internet-based resources such as the Monarch Initiative website (https://monarchinitiative.org/)[32] and Gene2Function (http://www.gene2function.org/search/)[33] may also provide useful information.

1.2 Gather additional information related to the following questions to further assess whether the variant is a good disease candidate or not from a protein function and structure point of view.

Q6) Is the variant of interest predicted to be damaging based on *in silico* prediction algorithms?
Q7) Does the human gene/protein of interest or its MO orthologs genetically or physically interact with genes/proteins previously linked to genetic diseases? If so, do these diseases have overlapping phenotypes with our patient of interest?
Q8) Has the three-dimensional structure of the protein of interest been solved or modeled? If so, where does the variant of interest map relative to its key functional domains?

Note: The following databases and tools can be useful to gather the answers for these questions **(A6-8)**. MARRVEL tool will be upgraded to incorporate this information in future updates[31].

A6) A number of variant pathogenicity algorithms have been developed by many research groups over the past ~15 years. More recent programs, including the two listed below, combine multiple variant pathogenicity prediction algorithms and machine learning approaches to generate a pathogenicity score. For more information on variant prediction algorithms and their performance, we refer the readers to Ghosh *et al*[8].

- CADD (Combined Annotation-Dependent Depletion): Integrative annotation tool built from more than 60 genomic features, which provides scores for human SNVs as well as short insertions and deletions. (cadd.gs.washington.edu)[11]
- REVEL (Rare Exome Variant Ensemble Learner): Combines multiple variant pathogenicity algorithms (MutPred, FATHMM, VEST, PolyPhen, SIFT, PROVEAN, MutationAssessor, MutationTaster, LRT, GERP, SiPhy, phyloP, and phastCons) to provide an integrated score for all possible human missense variants. (sites.google.com/site/revelgenomics)[34]

A7) Several tools have been developed to analyze genetic and protein-protein interactions based on MO publications as well as large-scale proteomics from multiple species screens.

- STRING (Search Tool for Recurring Instances of Neighboring Genes) (string-db.org)[35]: A database for known and predicted protein-protein interactions. It integrates genetic interaction and co-expression datasets as well as text-mining tools to identify genes and proteins that may function together in a variety of organisms.
- MIST (Molecular Interaction Search Tool) (fgrtools.hms.harvard.edu/MIST)[36]: A database that integrates genetic and protein-protein interaction data from core genetic MOs (yeast, *C. elegans*, *Drosophila*, zebrafish, frog, rat and mouse) and humans. Prediction of interactions inferred from orthologous genes/proteins (interlogs) are also displayed.

A8) Protein structures that have been solved by X-ray crystallography, nuclear magnetic resonance (NMR) and cryo-electron microscopy can be found in public databases including the PDB (Protein Data bank) (www.wwpdb.org) and EMDatabank (www.emdatabank.org)[37]. Although there is no single database for predicted/modeled protein structures, a number of algorithms including SWISS-MODEL (swissmodel.expasy.org)[38], Modeller (salilab.org/modeller)[39] and Phyre$_2$ (www.sbg.bio.ic.ac.uk/phyre2)[40] are available for users to perform protein modeling.

1.3 Communicate with your clinical collaborator to discuss the information you gathered from the informatics analysis in **1.1** and **1.2**. If you and your clinical collaborator both feel that the variant and gene of interest are good candidates to explain the phenotypes seen in the patient of interest, proceed to **Section 2**. If you have specific questions about the patient's genotype and phenotype, make sure to ask them before moving forward. If you feel the variant of interest is unlikely to explain the patient's phenotype of interest (e.g. identical variant found in high frequency in control population), you must discuss this with your clinical collaborators to determine whether the variant is really a good candidate that is worth investing time and effort.

Note: If your clinical collaborators can identify other patients who have similar genotypes and phenotypes with your patient of interest, this will significantly increase the likelihood that the variant of interest is pathogenic. We encourage the MO researchers to work closely with clinical researchers to search for additional patients with variants in a specific gene of interest. The following two tools, for example, allow one to search through a cohort of patients enrolled in diverse clinical studies to identify patients carrying identical or similar variants.

- Geno$_2$MP (Genotypes to Mendelian Phenotypes) (geno2mp.gs.washington.edu): A de-identified database of 9,650 individuals (as of Sep 2018) enrolled in the University of Washington Center for Mendelian Genomics study[41] that recruits patients suspected to have genetic disorders as well as their relatives. One can search for variants in a specific gene of interest and determine whether a specific variant is found in a patient with certain phenotypes (classified by the main organ system affected) or in unaffected family members (can be considered as 'control' for severe dominant disorders). If there are interesting patients in this database, one can use the "Contact" feature to reach out to the primary physicians who deposited the case via e-mail.
- GeneMatcher (www.genematcher.org): A matchmaking website for clinicians, basic researchers and patients who share interest in the same gene. Upon registration and submission of a gene of interest, GeneMatcher will provide the contact information of other submitters who expressed interest in the same gene. Since most users are clinicians and human geneticists, one can contact them via e-mail to see whether their patient's genotype and phenotype matches your patient of interest. Since GeneMatcher is part of Matchmaker Exchange (www.matchmakerexchange.org)[42], one can also search additional matchmaking databases around the world including Australian Genomics Health Alliance Patient Archive (mme.australiangenomics.org.au/#/home), Broad Matchbox (seqr.broadinstitute.org/matchmaker/matchbox), DECIPHER (decipher.sanger.ac.uk), MyGene2 (www.mygene2.org/MyGene2) and PhenomeCentral (phenomecentral.org) by gene submissions through GeneMatcher.

2. Gather existing genetic tools and establish new reagents to study a specific variant of interest

Once you determined that the variant of interest is a good candidate to pursue experimentally, the next step is to gather or generate reagents to perform *in vivo* functional studies. For functional studies described in this protocol, one will need a few key reagents. 1) UAS-Human cDNA transgenic strains that carry the reference or variant sequence, 2) a LOF allele of a fly gene of interest, and 3) a GAL4 line that can be used for rescue experiments.

2.1. Generation of UAS-human cDNA constructs and transgenic flies

2.1.1. Identify and obtain the appropriate human cDNA constructs. Many clones are available from the MGC (Mammalian Gene Collection)[43] and can be purchased from selected venders (genecollections.nci.nih.gov/MGC). Many cDNAs are available in Gateway compatible reagents[44], which simplifies the subcloning step. If the cDNAs is not included in the MGC or if one wishes to use a specific splice isoform not distributed by non-profit venders, one can looking into

commercial venders or use gene synthesis services. We typically select a cDNA corresponding to the longest isoform [(typically referred to as the canonical isoform in Enselmbl (useast.ensembl.org) or RefSeq (www.ncbi.nlm.nih.gov/refseq), two major databases that curate cDNA isoforms] if there are multiple options. One should especially be careful when a variant of interest only affects a subset of splicing isoforms.

Note: cDNAs may come in an "open (no stop codon)" or "closed (with endogenous or artificial stop codon)" format. Open clones allow C' of tagging of proteins when subcloned into a plasmid with C' tags (e.g. 3xHA tags for pUASg-HA.attB or pGW-HA.attB), whereas proteins will not be tagged if closed clones are subcloned into the same vector. While protein tags may offer biochemical (e.g. western blot) and cell biological (e.g. immunostaining) ways of monitoring the expression of the protein of interest, it may interfere with protein function in some cases.

2.1.2 Sub-clone the reference and variant cDNA into the *Drosophila* transgenic vector. The ϕC31-mediated transgenesis system is best suited for functional studies using human cDNAs since the reference and variant cDNAs can be integrated into the same location in the genome[45]. For this project, the MOSC *Drosophila* Core routinely use the pGW-HA.attB vector[46]. This is a Gateway compatible vector that contains 5xUAS sites, an *hsp70* promotor, C' 3xHA tag with a protein linker sequence, *tubulin α1* 3'UTR and mini-*white*$^{+}$ gene as a transgenesis marker. There are two altered *FRT* (*Flippase Recombination Target*) sites (*FRT5* and *FRT2*) flanking the open reading frame that can further be used to modify the transgene after genomic integration[47].

If the human cDNA is in Gateway compatible vectors (e.g. pDONR221, pDONR223, pENTR221, pENTR223.1), one can skip to **2.1.4** that explains LR reactions to subclone the cDNAs into pGW-HA.attB. If the human cDNA plasmids are not in a Gateway compatible vector, one can first subclone the fragments into a suitable entry vector (e.g. pDONR221) via the following protocol.

2.1.2a. Subclone the human cDNAs into a gateway compatible plasmid

2.1.2a.1 Perform an overhang PCR to introduce *attB1* and *attB2* arms. The forward primer should have the *attB1* sequence **5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTTCACC-3'** followed by the first 22 nucleotides of the target cDNA. The reverse primer should have the *attB2* sequence **5'-GGGGACCACTTTGTACAAGAAAGCTGGGTCCTA-3'** followed by the reverse complement of the last 25 nucleotides of your cDNA of interest. One can excluding the stop codon if you wish to "open" the clone to add a C' tag, or add a stop codon if you wish to "close" an open clone.

2.1.2a.2 Prepare a 100 µL Q5 PCR mix consisting of 50 µL Q5 mastermix (NEB #M0491), 36 µL milliQ water, 5 µL of each forward and reverse primers listed in **2.2.1** diluted to 10 µM, and 4 µL of target cDNA (150 ng/µl).

2.1.2a.3 Perform the PCR using standard Q5 mutagenesis protocol (NEB #M0491)

2.1.2a.4 Isolate the target cDNA with added homology arms via gel electrophoresis and gel extraction. Create 1% agarose gel and perform electrophoresis using standard methods. Cut out

the band that corresponds to the size of your cDNA plus the additional length of the homology arms. Extract DNA from the gel through standard methods. Commercial gel extraction kits are available from with several companies (Qiagen #28704).

2.1.2a.5 Perform a BP clonase reaction using standard Gateway cloning protocol (Invitrogen #11789)

2.1.2a.6 Transform the BP reaction mix into chemically competent *E coli* cells. Competent cells can be made in house or purchased from commercial venders (e.g. NEB #C2987H). Culture the transformant overnight on an LB plate containing appropriate antibiotics for colony selection. The next day select several colonies and grow them up in independent liquid cultures overnight.

2.1.2a.7 Isolate DNA from the overnight cultures and perform diagnostic restriction digests. Sanger sequence the positive clones to ensure that the cDNA is the correct sequence. We recommend generating glycerol stocks from the cultures that were positive for your desired sequence at this point.

2.1.3. Introduce the variant of interest into your Gateway plasmid with your reference human cDNA. There are several ways to perform this mutagenesis step including methods described in other JoVE protocols[48,49]. We have been using the Q5 site-directed mutagenesis system in our operation. Detailed protocol for this method can be found in the vendor's website (NEB #E0554S). In addition to validating the presence of the variant in the mutated plasmid, perform Sanger sequencing of the entire open reading frame (ORF) in order to make sure there are no additional variants introduced through this mutagenesis step.

Note: If the mutagenesis is not successful, we typically re-design the primer and repeat the experiment. If the mutagenesis fails again, we explore other methods such as QuikChange II Site Directed Mutagenesis system (Aligent #200523).

2.1.4 Subclone the reference and variant human cDNAs in the donor plasmid (Gateway plasmids with *attL1* and *attL2* sites) into the transgenic plasmid (e.g. pGW-HA.attB with *attR1* and *attR2* sites) via the LR clonase reaction (Thermo Fisher #11791100).

Note: There are UAS ϕC31 vectors that are designed for conventional restriction enzyme based subcloning (e.g. pUAST.attB[50]) if one prefers to subclone human cDNAs via traditional methods.

2.1.4 Inject the UAS-human cDNA constructs into flies expressing the ϕC31 integrase in their germline (e.g. vas-ϕC1, nos-ϕC31) (bdsc.indiana.edu/stocks/phic31/phic31_int.html). Microinjection can be performed in house, or can be sent to core facilities or commercial entities for transgenesis. Detailed protocol for generating transgenic flies can be found in the following book chapter[51].

Note: One needs to select a docking site for transgene insertion. Since it is convenient to have the human transgene on a chromosome that does not contain the fly ortholog of the gene of

interest, we typically use a 2nd chromosome docking site [VK37 (BDSC stock #24872, flybase.org/reports/FBst0024872.html)] when the fly ortholog is on the X, 3rd or 4th chromosomes and a 3rd chromosome docking site [VK33 (BDSC stock #24871, flybase.org/reports/FBst0024871.html)] when the fly ortholog is on the 2nd chromosome. A number of additional docking sites have been generated by several laboratories and are publically available from stock centers (bdsc.indiana.edu/stocks/phic31/phic31_attp.html , kyotofly.kit.jp/stocks/documents/phiC31.html)[50,52,53].

2.1.5. Establish stable transgenic strains from the injected embryos. We typically inject ~100-200 embryos per construct. A representative crossing scheme for a transgene insertion into a 2nd chromosome docking site (VK37) is depicted in **Figure 1A**. For basic *Drosophila* genetics information, we refer the readers to the following books[54,55].

2.2 Obtain or generate a T2A-GAL4 line that facilitates rescue-based functional assays (see **Figure 2** and **Section 3.1**). This line will serve two purposes. First, most T2A-GAL4 lines tested behave as strong LOF alleles by functioning as a gene trap allele. Second, T2A-GAL4 lines function as a GAL4 driver that allows expression of UAS constructs (e.g. UAS-GFP, UAS-human cDNAs) in the pattern of the fly gene of interest[56,57] (**Figure 2A-C**).

2.2.1 Search public stock collections for available T2A-GAL4 lines. Through the *Drosophila* Gene Disruption Project (GDP)[58], ~1,000 T2A-GAL4 lines have been generated[59]. These strains are currently available from the Bloomington *Drosophila* Stock Center (BDSC) and are searchable through both the GDP (flypush.imgen.bcm.tmc.edu/pscreen) and BDSC (bdsc.indiana.edu) websites.

2.2.2. If a T2A-GAL4 line for your fly gene of interest is not available, check if a suitable coding intronic MiMIC (*Minos* mediated Integration Cassette) line is available for conversion into a T2A-GAL4 line using recombinase mediated cassette exchange (RMCE)[60] (**Figure 2A**). RMCE allows intronic MiMIC elements that are in between two coding exons to be converted into a T2A-GAL4 line through injection (an example of a crossing scheme is shown in **Figure 1B**) or series of crosses as described in detail in the following papers[57,59].

2.2.3. If a T2A-GAL4 is not available and an appropriate coding intronic MiMIC does not exist, explore the possibility of generating a T2A-GAL4 line via the CRIMIC (CRISPR-mediated Integration Cassette) system as described in Lee *et al.*[59]. This methodology uses CRISPR-mediated DNA cleavage and homology directed repair (HDR) to integrate a MiMIC-like cassette into a coding intron in a gene of interest.

Note: Not all genes can be tagged using a T2A-GAL4 system. For genes that lack introns or only have small (<100bp) coding introns, one can attempt to knock-in a GAL4 transgene into the fly gene of using the CRISPR/Cas9 system using HDR as described in the following papers[20,61,62]. Alternatively, if the gene of interest have previously characterized mutants, one can attempt to perform rescue experiments using these pre-existing alleles and ubiquitous or tissue-specific GAL4 drivers.

3. Perform Functional Analysis of the human variant of interest *in vivo* in *Drosophila*

Perform a rescue-based analysis (**Section 3.1**) as well as over-expression studies (**Section 3.2**) using the tools gathered or generated in **Section 2** to assess the functional consequence of the variant of interest *in vivo* in *Drosophila*. The two approaches are complementary to one another.

3.1. Functional analysis through rescue based experiments.

Heterologous rescue-based experiments in *Drosophila* using human proteins determine whether the molecular function of the two orthologous genes have been conserved over ~500 million years of evolution, and further assess the function of the variant in the context of the human protein[63]. Although a systematic analysis studying hundreds of gene pairs has not been reported, several dozen human and mammalian (e.g. mouse) genes have been able to replace the function of *Drosophila* genes[13].

3.1.1 In the rescue-based approach, we first determine whether there are obvious scorable and reproducible phenotype in LOF mutants in the fly ortholog before assessing the function of variants. Previous literature on the fly gene is the first place to mine for data and can be found using databases such as FlyBase (flybase.org/) and PubMed (www.ncbi.nlm.nih.gov/pubmed/). Additional databases such as MARRVEL (marvel.org), Monarch Initiative (monarchinitiative.org/), and Gene2Funcion (http://www.gene2function.org) are also useful in gathering this information. If the T2A-GAL4 allele is the first mutation to be characterized for a specific gene, one should perform a global survey of scorable phenotypes in homozygous and hemizygous (T2A-GAL4 allele over a molecularly defined chromosomal deficiency; e.g. bdsc.indiana.edu/stocks/df/index.html) animals. These include lethality, sterility, longevity, morphological (e.g. size and morphology of the eye) or behavioral phenotypes (e.g. courtship, flight, climbing and bang sensitivity defects). More sophisticated phenotypes such as neurological defects measured by electrophysiological recordings can also be used as long as they are highly reproducible and specific. Functional studies using electroretinogram (ERG) are described in **3.2.3**.

3.1.2 Once a scorable phenotype is identified in the fly LOF mutant, test whether the reference human cDNA can replace the function of the fly ortholog. If this “humanization” of the fly gene is successful, we now have a platform to compare the efficiency of the variant of interest compared to the reference counterpart. The rescue seen with reference human cDNA does not have to be perfect. Partial rescue of the fly mutant phenotype using a human cDNA still provides a reference point to perform comparative studies using the variant human cDNA strain.

3.1.3 Using the assay system selected in **3.1.2**, compare the rescue observed with the reference human cDNA to the rescue observed with the variant human cDNA to determine if the variant of interest has functional consequences.

3.1.4. If the variant is found to be a LOF allele, one can further compare the expression and intracellular localization of the reference and variant protein of interest *in vivo*, especially if the UAS-transgenes were generated from an ‘open’ clone and have a C-terminal tag that can be used

to image the proteins via western blot, immunofluorescence staining or other methods. If the transgenes were generated from a 'closed' clone, one can look into commercial antibodies raised against the human protein of interest and assess whether these reagents can be used to detect these proteins in a fly tissue.

3.2 Functional analysis through over-expression studies

Ubiquitous or tissue-specific over-expression of human cDNAs in otherwise wild-type flies can provide information that is complementary to the rescue-based experiments. While rescue-based assays are primarily designed to detect LOF variants (amorphic, hypomophic), over-expression based assays may reveal gain-of-function (GOF) variants that may be missed (hypermorphic, antimorphic, neomorphic).

3.2.1 Select a set of GAL4 drivers to over-express the human cDNAs of interest. A number of ubiquitous, tissue and stage specific GAL4 drivers are available from public stock centers (e.g. bdsc.indiana.edu/stocks/gal4/index.html, kyotofly.kit.jp/stocks/documents/GAL4.html), some of which are more frequently used than others. A large collection of GAL4 lines and related resources are being constructed for which we refer the readers to the following papers[56,57,59]. Upon obtaining these drivers, make sure to validate drivers with a reporter line (e.g. UAS-GFP) to confirm their expression pattern upon receiving the stocks from a stock center.

3.2.2 Express the reference and variant human cDNAs using the same driver under the same condition (e.g. temperature) and ascertain if there is a difference between them. If a phenotype is only seen in the reference but not in the variant line, the variant may be an amorphic or a strong hypomorphic allele. If the phenotype is seen in both genotypes, but the reference causes a stronger defect, the variant may be a mild to weak hypomorphic allele. If the reference does not show a phenotype, or only exhibits a weak phenotype, but the variant shows a strong defect, the variant may be a GOF allele. We recommend the readers first focus on ubiquitous drivers and easily scorable phenotypes (lethality, sterility, morphological phenotypes), and move on to tissue specific drivers and more specific phenotypes. We also recommend the readers to test the flies in different temperatures ranging between 18°C to 29°C because the UAS/GAL4 system is known to be temperature-dependent[64,65]. Typically, the expression of UAS transgenes are higher at higher at higher temperatures.

3.3 Perform additional functional studies related to the genes/protein of interest. In addition to examining general defects, one can select an assay system to probe into the molecular function of the gene and variant. In one of the example discussed under "Representative Results" section (*TBX2* case), we used ERG recordings to determine the effect of the variant on photoreceptor function since the fly gene of interest (*bifid*) had been studied extensively in the context of visual system development. Here, we describe a general outline of how to carry out such experiment in flies that over-express a reference or variant form of a human protein of interest in photoreceptors. Detailed protocol for ERG in *Drosophila* can be found in the following papers[66–68].

3.3.1 Set up crosses to generate flies to test for functional defects in the visual system. One can use Rh1-GAL4 to drive the reference or variant UAS-human cDNA transgenes in the R1-R6 photoreceptors by a single cross (Rh1-GAL4 virgin females x UAS-human cDNA males) to obtain the flies for ERG testing (Rh1-GAL4/+; UAS-human cDNA/+). We typically cross 3-5 virgin females to 3-5 male flies in a single vial and transfer the crosses every 2-3 days to have many animals eclosing from a single cross. The crosses are kept in an incubator set at the experimental temperature.

3.3.2 Once flies begin to eclose (at 25°C, ~10 days after setting the initial cross), separate them from the remaining pupae and place them in fresh vials. Place them back into the incubator set at the experimental temperature for an additional 3 days. We recommend recording ERGs on 3-5 day old flies since flies that are newly eclosed may still have large fluctuations in their ERG signal. If one wants to examine an age-dependent phenotype, these flies can be aged for several weeks as long as they are regularly (e.g. every ~5 days) transferred to a new vial to avoid the flies from drowning in wet food.

3.3.3 Prepare the flies for ERG recording by first anesthetizing the flies using CO_2 or placing them into a vial on ice. Gently glue one side of the fly onto a glass microscope slide to immobilize them. Multiple reference and variant flies can be glued on to a single slide. Place all flies in approximately the same orientation with one eye being accessible for the recording electrode. Be careful not to get glue on the eye and to leave the proboscis free.

3.3.4 Prepare the electrodes: Place a glass capillary into a needle puller (e.g. NARISHIGE Model PP-830) and switch on the filament. As soon as the weight drops, turn off the puller and detach the pulled capillary tube from the machine. This procedure will break the capillary tube to obtain two sharp tapered electrodes. The settings of the puller should be adjusted to obtain sharp tapered ends on the capillaries according to your system.

3.3.5 Fill the capillaries with saline solution (100 mM NaCl), making sure there are no air bubbles. Slide the glass capillaries over the silver wire electrodes (both the recording electrode and reference electrode, see **Figure 4**) and secure the capillaries in place.

3.3.6 Configure the stimulator and amplifier. Detailed set up can be found in Lauwers *et al.*[67] Our set up consists of the following equipment:

- Iso-Dam Isolated Biological Amplifier (World Precision Instruments, Sarasota, FL, USA): Set the amplifier to 0.1 Hz high pass filter, 300 Hz low pass filer, and 100 gain.
- S48 Stimulator (Astro-Med Inc. GRASS Instrument Division; West Warwick, RI, USA): Set the stimulator to 1 s period, 500 ms pulse width, 500 ms pulse delay, run mode, and 7 amplitude.
- Light source: We use a halogen light source (ACE Light Source, SCHOTT North America Inc., Southbridge, MA, USA) to stimulate the fly photoreceptors
- Axoscope 10.5 data acquisition software (Molecular Devices, San Jose, CA, USA): Create a stimulation protocol with acquisition model “fixed length events” and 20 s duration.

3.3.7 Acclimate the flies to complete darkness before initiating the ERG recordings. We typically place the flies into complete darkness for at least 10 minutes before beginning the experiment. Place the slide containing the flies onto the recording apparatus.

Note: Since flies cannot see red light, one can use a red light source during the period of dark habituation.

3.3.8 Move the micromanipulators carrying the reference and recording electrodes to a point that is close to the fly of interest on the slide. Watch the tip of the electrode and carefully place the reference electrode into the thorax of the fly. The exact position of this reference electrode does not have a major impact on the ERG signal. Then, place the recording electrode on the surface of the eye. ERG is a field recording so the recording electrode should be placed at the surface of the eye. The perfect amount of pressure will cause a small dimple, but should not penetrate the eye.

3.3.9 Turn off all lights for another 3 minutes to acclimate the flies again to the dark environment. In the Axoscope software, press play. If using a halogen light source with a manual shutter, turn on the light source at this point with the shutter closed (flies are still in dark). Next press record in the Axoscope and expose the fly eyes to light by opening and closing the shutter every 1 second for the 20 second duration of a single run. We control the on/off of the halogen light source manually but this can be programmed to have it automated using a white LED light source. In our experience, however, we have obtained much more robust and reliable ERG by using a halogen light source compared to a white light LED, likely due to the broader light spectrum emitted from the halogen light source.

3.3.10 Record ERGs from all of the flies that are mounted on the glass slide. Typically, we perform ERGs from 15 flies per genotype per condition. Parameters that can be altered to find a condition that shows robust differences between reference and variant cDNAs may include temperature, age, or environmental conditions (e.g. reared in light-dark cycle or constant light/darkness).

3.3.11 Perform data analysis: Compare the ERGs from the reference, variant, and controls to determine if there are differences. ERGs can be assessed for changes in on-transients, depolarization, off-transients, and repolarization[69] (**Figure 4B**). Depolarization and repolarization reflects the activation and inactivation of the phototransduction cascade within the photoreceptors, whereas the on- and off- transients are measures of the activities of post-synaptic cells that receive signals from the photoreceptors. Decreased amplitude and altered kinetics of repolarization are often associated in defects with photoreceptor function and health, whereas defect in on- an off-transients are found in mutants with defective synapse development, function or maintenance[70].

Note: Upon identification of differences in ERG phenotypes with over-expression of reference versus variant human cDNAs, one can further determine whether this electrophysiological phenotype is associated with structural and ultrastructural defects in photoreceptors and its synapses by performing histological analysis as well as transmission electron microscopy. Further

discussion on interpretation of ERG defects and structural/ultrastructural analysis can be found in the following article[69].

Representative Results:

1. Functional Study of a *de novo* missense variant in *EBF3* linked to neurodevelopmental phenotypes

In a 7-year-old male with neurodevelopmental phenotypes including hypotonia, ataxia, global developmental delay and expressive speech disorder, physicians and human geneticists at the National Institutes of Health Undiagnosed Diseases Project (UDP) identified a *de novo* missense variant (p.R163Q) in *EBF3 (Early B-Cell Factor 3*)[15], a gene that encodes a COE (Collier/Olfactory-1/Early B-Cell Factor) family transcription factor. This case was submitted to the UDN MOSC in March 2016 for functional studies of this variant. To assess whether this gene was a good candidate for this case, the MOSC gathered human genetic and genomic information from OMIM (www.omim.org/), ClinVar (www.ncbi.nlm.nih.gov/clinvar/), ExAC [exac.broadinstitute.org/ (now expanded to gnomAD, gnomad.broadinstitute.org/)], $Geno_2MP$ (geno2mp.gs.washington.edu/Geno2MP/#/), DGV (dgv.tcag.ca/dgv/app/home), and DECIPHER (decipher.sanger.ac.uk/). In addition, we identified the orthologous genes in key MO species using the DIOPT tool (www.flyrnai.org/cgi-bin/DRSC_orthologs.pl), and further obtained gene expression and phenotypic information from individual MO databases [e.g. Wormbase (www.flyrnai.org/cgi-bin/DRSC_orthologs.pl), FlyBase (flybase.org/), ZFIN (zfin.org/) and MGI (www.informatics.jax.org/)]. Our gene variant interpretation methodology used for *EBF3* and other pioneering studies formed the basis for the later development of the MARRVEL resource (marrvel.org/) in 2017[30].

The information gathered from this methodology indicated *EBF3* was not associated with any known human genetic disorder at the time of analysis, and we concluded that the p.R163Q variant was a good candidate for this case based on the following information. (1) This variant has not been previously reported in control population databases (ExAC) and disease population database ($Geno_2PM$), indicating that this is a very rare variant. (2) Based on ExAC, pLI (probability of LOF intolerance) score of this gene is 1.00 (pLI score ranges from 0.00 to 1.00). This indicates that there is a selective pressure against LOF variants in this gene in the general population and suggests that haploinsufficiency of this gene may cause disease. For more information on pLI score and its interpretation, please refer to the accompanying MARRVEL tutorial article in JoVE[31] as well as related papers[30,71]. (3) The p.R163Q variant is located in the evolutionarily conserved COE DNA binding domain of this protein, suggesting that it may affect DNA binding or other protein function. (4) The p.R163 residue is evolutionarily conserved from *C elegans* and *Drosophila* to human, suggesting that it may be critical for protein functional across species. (5) *EBF3* orthologs have been implicated in neuronal development in multiple MO[72] including *C elegans*[73], *Drosophila*[74], Xenopus[75]and mice[76]. (6) During brain development in mice, *Ebf3* was

shown to function downstream of *Arx* (*Aristaless-related homebox*)[77], a gene known to be associated with several epilepsy and intellectual disability syndromes in human[78]. Hence, these data together suggested that *EBF3* is highly likely to be crucial to human neurodevelopment and that the p.R163Q variant may have functional consequences.

To assess whether p.R163Q affects EBF3 function, a T2A-GAL4 line for *knot (kn)*, the fly ortholog of human *EBF3*[79] was generated via generated via RMCE of a coding intronic MiMIC cassette[15]. The $kn^{T2A\text{-}GAL4}$ line was recessive lethal and failed to complement the lethality of a classic *kn* allele ($kn^{col\text{-}1}$) as well as a molecularly defined deficiency that covers *kn [Df(2R)BSC429]*[80]. Expression pattern of the GAL4 also reflected previously reported patterns of *kn* expression in the brain as well as in the wing imaginal disc[15]. UAS transgenic flies were generated to allow the expression of reference and variant human EBF3 cDNA as well as a wild-type fly *kn* cDNA. All three proteins were tagged with a C' 3xHA tag. Importantly, UAS-wild-type fly *kn* (kn^{+}) or reference human *EBF3* ($EBF3^{+}$) transgenes rescued the lethality of $kn^{T2A\text{-}GAL4}/Df(2R)BSC429$ to a similar extent (**Figure 3C, left panel**)[81]. In contrast, UAS-human *EBF3* transgene with the p.R163Q variant ($EBF3^{p.R163Q}$) was not able to rescue this mutant, suggesting that the p.R163Q variant affects EBF3 function *in vivo*[15]. Interestingly, when assessed using an anti-HA antibody, the $EBF3^{p.R163Q}$ protein was successfully expressed in the fly tissues and its levels and subcellular localization (primarily nuclear) was indistinguishable from that of $EBF3^{+}$ and Kn^{+}. This suggests that the variant is not causing a LOF phenotype due to protein instability or mis-localization. To further assess whether the p.R163Q variant affected the transcriptional activation function of *EBF3*, a luciferase based reporter assay was performed in HEK293 cells[15]. This experiment in cultured human cells revealed that the $EBF3^{p.R163Q}$ variant failed to activate transcription of the reporter constructs, supporting the LOF model obtained from *Drosophila* experiments.

In parallel to the experimental studies, collaborations with physicians, human geneticists, and genetic counselors at BCM identified two additional individuals with similar symptoms. One patient carried the identical p.R163Q variant, and another patient varied a missense variant that affected the same residue (p.R163L). The p.R163L variant also failed to rescue the fly *kn* mutant[93] suggesting that this allele also affected *EBF3* function. Interestingly, this work was published back-to-back with two independent studies that reported additional individuals with *de novo* missense, nonsense, frameshift and splicing variants in *EBF3* linked to similar neurodevelopmental phenotypes[82,83]. Subsequently, three additional papers were published reporting additional cases of *de novo EBF3* variants and copy number deletion[84–86]. This novel neurodevelopmental syndrome is now known as the 'Hypotonia, Ataxia, and Delayed Development Syndrome (HADDS, OMIM #617330)' in the Online Mendelian Inheritance in Man (OMIM, www.omim.org), an authoritative database for genotype-phenotype relationships in human.

2. Functional Study of a dominantly inherited missense variant in *TBX2* linked to a syndromic cardiovascular and skeletal developmental disorder

In a small family with affected with overlapping spectrum of craniofacial dysmorphisms, cardiac anomalies, skeletal malformations, immune deficiency, endocrine abnormalities and developmental impairments, the UDN Duke Clinical Site identified a missense variant (p.R20Q) in *TBX2* that segregates with disease phenotypes[87]. Three (son, daughter and mother) out of four family members are affected by this condition, and the son exhibited the most severe phenotype. Clinically, he met a diagnosis of 'complete DiGeorge syndrome', a condition that is often caused by haploinsufficiency of *TBX1*. While there were no mutation identified in *TBX1* in this family, the clinicians and human geneticists focused on a variant in *TBX2* since previous studies in mice showed that these genes have overlapping functions during development[88]. TBX1 and TBX2 both belong to T-box (TBX) family of transcription factors that can act as transcriptional repressors as well as activators depending on the context. Previously, variants in 12 out of 17 members of the *TBX* family genes were linked to human diseases. The MOSC decided to experimentally pursue this variant based on the following information gathered through MARRVEL and other resources. (1) This variant was reported only once in a cohort of ~90,000 'control' individuals in gnomAD (note that this variant was filtered out in a default view, likely due to low coverage reads). Considering the milder phenotypic presentation of the mother, this still can be considered as a very rare variant that may be responsible for the disease phenotypes. (2) The pLI score of *TBX2* in ExAC/gnomAD are 0.96/0.99 which is high (Max for pLI is 1.00). In addition, the o/e (observed/expected) LOF score in gnomAD is 0.05 (only 1/18.6 expected LOF variant is observed in gnomAD). These numbers suggest that LOF variants in this gene are selected against in the general population. (3) The p.R20 is evolutionarily conserved from *C elegans* and *Drosophila* to human, suggesting that this may be an important residue for TBX2 function. (4) Multiple programs predict that the variant is likely damaging. Polyphen: Possibly/Probably Damaging, SIFT: Deleterious, CADD Score: 24.4, REVEL Score: 0.5. (5) MO mutants exhibit defects in tissues affected in patients (e.g. knockout mice exhibit defects in cardiovascular system, digestive/alimentary systems, craniofacial, limbs/digit). Hence, together with the biological links between *TBX1* and *TBX2* and the phenotypic links between our patients and DiGeorge Syndrome, we decided to perform functional studies of variants in this gene using *Drosophila*.

To begin to assess whether the p.R20Q variant affects *TBX2* function, we first generated a T2A-GAL4 line in *bifid* (*bi*), the *Drosophila* ortholog of human *TBX2*, via RMCE of a coding intronic MiMIC (**Figure 2**)[87]. This allele, $bi^{T2A\text{-}GAL4}$, was recessive pupal lethal and behaved as a strong LOF mutant similar to previously reported *bi* LOF alleles (e.g. bi^{D2}, bi^{D4}) (**Figure 2E**). We were able to rescue the lethality of $bi^{T2A\text{-}GAL4}$ as well as other *bi* alleles tested using an ~80kb genomic rescue construct carrying the entire *bi* locus, indicating that these are indeed clean LOF alleles. The expression pattern of GAL4 in the $bi^{T2A\text{-}GAL4}$ line also matched well with previously reported patterns of *bi* expression in multiple tissues including in the wing imaginal disc (**Figure 2D**). In parallel, we generated UAS-transgenic lines for *TBX2* carrying the reference or variant (p.R20Q) sequences. Unfortunately, both transgenes were not able to rescue lethality of the $bi^{T2A\text{-}GAL4}$ line. Importantly, we also found that a wild-type fly *UAS-bi* transgene also failed to rescue the $bi^{T2A\text{-}}$

^{GAL4} allele, likely due to the dosage-sensitivity of this gene. Indeed, over-expression of *UAS-bi*$^{+}$ as well as *UAS-TBX2*$^{+}$ and *UAS-TBX2*$^{p.R20Q}$ caused some degree of lethality when overexpressed in a wild-type animal. We decided to use this toxic effect of *bi/TBX2* over-expression as a functional assay to assess whether the p.R20Q affects TBX2 function. Since the Drosophila *bi* gene has been extensively studied in the context of the visual system (gene is also known as [*optomotor blind* (*omb*)), we decided to primarily focus on phenotypes related to the eye. When we expressed reference *TBX2* using an *ey-GAL4* driver that expresses UAS-transgenes in the eye as well as in parts of the brain relevant to the visual system, we observed ~85% lethality (**Figure 3C, right panel**) and significant reduction of eye size (**Figure 4B**). This phenotype was stronger than the phenotype observed when a wild-type fly *UAS-bi* transgene was expressed, suggesting that the human TBX2 causes is more detrimental to the fly when overexpressed. Interestingly, the p.R20Q TBX2 was less potent in causing lethality (**Figure 3C, right panel**) as well as inducing a small eye phenotype (**Figure 4B**) using the same driver under the identical condition[87], suggesting the variant affects protein function. Moreover, when we assessed the function of photoreceptors over-expressing reference and variant *TBX2* using a different GAL4 driver [*Rhodopsin 1 (Rh1)-GAL4* that specifically expresses UAS transgenes in R1-R6 photoreceptors), we also observed that variant TBX2 exhibited a much milder ERG phenotype compared to reference TBX2 (**Figure 4B**)[87]. Interestingly, most of the p.R20Q TBX2 protein was still found in the nucleus similar to the reference protein, suggesting that the variant did not affect nuclear localization. When we performed a luciferase based transcription repression assay in HEK293T cells, we found that the p.R20Q was not able to effectively repress transcription of a reporter construct with palindromic T-box sites[87]. In addition, we observed a decrease in protein levels of TBX2$^{p.R20Q}$ compared to TBX2^{+}, suggesting that the variant may affect translation or protein stability of TBX2, which in turn affects its abundance within a cell.

In parallel to these experimental studies, we attempted to identify additional patients with rare variants in *TBX2*. Through GeneMatcher, we identified an 8-year-old boy with a *de novo* missense (p.R305H) variant from an unrelated family who exhibited many of the features found in the first family[87]. Additional functional studies in *Drosophila* and human cell line revealed that the p.R305H variant also affects TBX2 function and protein levels, strongly suggesting that defect in this gene is likely to underlie many of the phenotypes found in the two families. This disorder has been recently curated as 'Vertebral anomalies and variable Endocrine and T-cell Dysfunction (VETD, OMIM #618223)' in OMIM. Identification of additional individuals with functional variants in *TBX2* with overlapping phenotypes will be critical to establish the full spectrum of genotype-phenotype relationship for this gene in human disease.

Discussion:

Experimental studies using *Drosophila melanogaster* provides a robust assay system to assess functional consequences of disease associated human variants, thanks to the large body of knowledge and diverse genetic tools that have been generated by many researchers in the fly field over the past century[89]. Just like any other experimental systems, however, it is important to acknowledge the caveats and limitation when using this system.

Caveats associated with data mining

Although the first step in this protocol is to mine databases for information pertaining to a gene of interest, it is important to use this information as a starting point and not as solid evidence. For example, although *in silico* prediction of variant function provides valuable insights, these data should always be interpreted with caution. There are some instances in which all major algorithms predict that a human variant is benign, yet functional studies in *Drosophila* clearly demonstrated the functionality of such variant[24]. Similarly, although protein-protein interaction, co-expression and structural modeling data are all insightful pieces of information, there may be pseudo-positive and pseudo-negative information present in these large 'omics' data sets. For example, some of the previously identified or predicted protein-protein interactions may be artificial or only seen in certain cell or tissue types. In addition, there may be many false negative interactions that are not captured in these data sets since certain key protein-protein interactions are transient (e.g. enzyme-substrate interactions). Experimental validation is critical to demonstrate that certain genes or proteins genetically or physically interact *in vivo* and in the biological context of interest. Similarly, structures predicted based on homology modeling should only be treated as a 'model' rather than a concrete structure. Although this information could be useful if one finds that an amino acid of interest is present in a structurally important part of the protein, negative data does not rule out the possibility that the variant may be functional. Finally, some of the previously reported genotype-phenotype information may also need to be treated with caution since some information archived in public database may not be accurate. For example, some information in MO databases are based on experiments that have been well controlled and performed rigorously, whereas others may have been one of many hits that are described in a large screen paper without additional follow-up studies with stringent controls.

'Humanization' experiments using T2A-GAL4 strategy may not always be successful

While rescue and over-expression based functional studies using human cDNAs allows assessments of variants in the context of the human protein, this approach is not always successful. If a reference human cDNA cannot rescue the fly mutant phenotype, there are two possible explanations. The first possibility is that the human protein is nonfunctional or has significantly reduced activity in the context of a fly cell. This could be due to reduced protein expression, stability, activity and/or localization, or could be due to the lack of compatibility with fly proteins that work in a multi-protein complex. Since the UAS/GAL4 system is temperature sensitive, one can raise the flies at a relatively high temperature (e.g. 29°C) to see if one may be able to see a rescue in this condition. In addition, one can also generate a UAS-fly cDNA construct and transgene as a positive control. If the variant of interest affects a conserved amino acid, the analogous variant can be introduced into the fly cDNA for functional study of the variant in the

context of the fly ortholog. Although this is not absolutely necessary, it greatly helps the study in case the experiments using human cDNA transgenic line gives negative or inconclusive results (**Figure 3**). The second possibility is that the expression of the human protein causes some sort of cellular or organism level toxicity. This could be due to an antimorphic effect (e.g. acting as a dominant negative protein), hypermorphic effect (e.g. too much activity), or neomorphic effect (e.g. gain of toxic function such as protein aggregation). In this case, keeping the flies in a low temperature (e.g. 18°C) may alleviate some of these problems. Importantly, if the human cDNA causes a gain of toxic function phenotype, we can take advantage of this and use this specific phenotype to assess the variant function as described in Section 3. Finally, there are some scenarios in which the over-expression of a fly cDNA may not rescue the fly T2A-GAL4 line as we have seen in the TBX2 example, likely due to the strict dosage dependence of the gene product. To avoid the over-expression of a protein of interest, one can modify the fly gene of interest via CRISPR or engineer a genomic rescue construct that contains the variant of interest and perform rescue experiments using a LOF allele[21]. For small genes, one can also consider 'humanizing' the fly genomic rescue construct to test human variants that affects non-conserved amino acids[24].

Things to note when interpreting negative and positive results

If both the reference and variant human cDNAs rescues the fly mutant phenotypes to a similar degree, and there is no difference observed in all conditions tested, we conclude that the variant is functionally indistinguishable in *Drosophila in vivo*. It is important to note that this information is not sufficient to rule out that the variant of interest is non-pathogenic since the *Drosophila* assay may not be sensitive enough or may not capture all potential functions of the gene/protein of interest that matter in humans. Positive data, on the other hand, is a strong indication that the variant has functional consequences, but is not sufficient to claim pathogenicity. American College of Medical Genetics and Genomics (ACMG) has published a set of standards and guidelines to classify variants in human disease associated genes into "benign", "likely benign", "variant of unknown significance (VUS)", "likely pathogenic" and "pathogenic"[90]. Although this classification only applies to established disease-associated genes and not directly applicable to variants in 'genes of uncertain significance (GUS)', we strongly encourage all individuals who are involved in human variant functional studies to read and adhere to this guideline when reporting variant function.

Extracting useful biological information when MO phenotypes do not 'model' the human disease condition

It is important to keep in mind that over-expression based functional assays have their own limitations, especially since some of the phenotypes being scored may have little relevance to the disease condition of interest. Similarly, the phenotypes that are being assessed through rescue experiments may not have any direct relevance to the disease of interest. Since these experiments are conducted outside the endogenous contexts in an invertebrate system, they should not be considered as a disease models but rather as a gene functional test using a 'living test tube'. Scorable phenotypes used in rescue experiments can often provide biological insights into the disease conditions. The concept of 'phenologs (non-obvious homologous phenotypes)'

(www.phenologs.org)[91] can be used to further determine the underlying molecular connection between the *Drosophila* and human phenotypes. For example, morphological phenotypes in the fly wing are excellent phenotypic readouts for defects in Notch signaling pathway, an evolutionarily conserved pathway linked to many congenital disorders including cardiovascular defects in humans[62]. By understanding the molecular logic behind certain phenotypes in *Drosophila*, one may identify hidden biological links between genes and phenotypes in humans that have yet to be understood.

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Disclosures:
The authors have nothing to disclose.

Online Resources:

Variant function prediction algorithms
- PolyPhen-2: http://genetics.bwh.harvard.edu/pph2
- SIFT: https://sift.bii.a-star.edu.sg
- CADD: https://cadd.gs.washington.edu
- PROVEAN: http://provean.jcvi.org/index.php
- MutationTaster: http://www.mutationtaster.org
- REVEL https://sites.google.com/site/revelgenomics

Rare and undiagnosed disease research consortiums
- UDN: https://undiagnosed.hms.harvard.edu
- RDMM: http://www.rare-diseases-catalyst-network.ca
- IRUD: https://irudbeyond.nig.ac.jp/en/index.html
- SOLVE-RD: http://solve-rd.eu
- Australian Functional Genomics Network: https://www.functionalgenomics.org.au

Integrative database for human and model organism Information
- MARRVEL: http://marrvel.org
- Monarch Initiative: https://monarchinitiative.org
- Gene2Function: http://www.gene2function.org
- Phenologs: http://www.phenologs.org

Human Genetic and Genomics Databases
OMIM: https://www.omim.org/)

ClinVar: https://www.ncbi.nlm.nih.gov/clinvar/)
ExAC: http://exac.broadinstitute.org/
gnomAD: http://gnomad.broadinstitute.org/
Geno$_2$MP: http://geno2mp.gs.washington.edu/Geno2MP/#/
DGV: http://dgv.tcag.ca/dgv/app/home
DECIPHER: https://decipher.sanger.ac.uk/

Ortholog Identification Tool
DIOPT: https://www.flyrnai.org/cgi-bin/DRSC_orthologs.pl

Model Organism Databases and Pubmed
Wormbase (*C elegans*): https://www.flyrnai.org/cgi-bin/DRSC_orthologs.pl
FlyBase (*Drosophila*): http://flybase.org
ZFIN (Zebrafish): https://zfin.org
MGI (Mouse): http://www.informatics.jax.org
Pubmed: https://www.ncbi.nlm.nih.gov/pubmed/

Genetic and protein interaction databases
STRING: https://string-db.org
MIST: http://fgrtools.hms.harvard.edu/MIST/

Protein structure databases and modeling tools
WWPBD: http://www.wwpdb.org
SWISS-MODEL: https://swissmodel.expasy.org/
Modeller: https://salilab.org/modeller/
Phyre^2: http://www.sbg.bio.ic.ac.uk/phyre2

Patient matchmaking platforms
Matchmaker Exchange: http://www.matchmakerexchange.org
GeneMatcher: https://www.genematcher.org
AGHA Archive https://mme.australiangenomics.org.au/#/home
matchbox: https://seqr.broadinstitute.org/matchmaker/matchbox
DECIPHER: https://decipher.sanger.ac.uk
MyGene^2: https://www.mygene2.org/MyGene2
Phenome Central: https://phenomecentral.org

Human transcript annotation and cDNA clone information
Mammalian Gene Collection: https://genecollections.nci.nih.gov/MGC
Ensembl: http://useast.ensembl.org
Refseq: http://www.ncbi.nlm.nih.gov/refseq

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Drosophila Stocks for UAS-human cDNA transgenesis		
VK33 (3rd chromosome) injection line	BDSC	#24871
VK37 (2nd chromosome injection) line	BDSC	#24872
Plasmid DNA		
pDONR221	Thermo Fisher	#12536-017
pGW-HA. attB	Gift from Drs. Johannes Bischof and Konrad Basler (Bischof et al., 2013 PNAS)	
Molecular biology kits and reagents		
Q5 Polymerase kit	NEB	#M0491
BP Clonase kit	Thermo Fisher	#11789020
LR Clonase II Enzyme kit	Thermo Fisher	#11791100
PureLink Gel Extraction Kit	Thermo Fisher	#K210012
Quick Change II Mutagenesis kit	Agilent	#200523
Agarose (molecular biology grade)	Sigma-Aldrich	#A2790
QIAprep Spin Miniprep Kit	Qiagen	#27104
DH5α	Thermo Fisher	#18265017
Electroretinogram Rig related equipment		
ISO-DAM Isolated Biologic Amplifier	LabX	#R150358
Square Pulse Stimulator	Astro-Med	#S48
Axon pCLAMP 10 Data Software Package	Molecular Devices	N/A

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Figure Legends:

Figure 1: Injection and crossing scheme to generate UAS-human cDNA and T2A-GAL4 lines. **(A)** Generation of UAS-human cDNA transgenes through microinjections and crosses. Crossing scheme to integrate the transgenes into a 2nd chromosome docking site (VK37) using male flies in the 1st and 2nd generation are shown as an example. Upon injection of the human cDNA ϕC31 transgenic construct (pGW-HA.attB) into early embryos that contain a germline source of ϕC31 integrase (labeled with both 3xP3-GFP and 3xP3-RFP) and VK37 docking site [labeled with a *yellow*$^+$ (*y*$^+$) marker], one can follow the transgenic events with the *white*$^+$ (*w*$^+$) minigene that is present in the transgenic vector. We recommend the readers to cross out the ϕC31 integrase by selecting against flies with GFP and RFP. The final stable stock can be kept as homozygotes or as a balanced stock if the chromosome carries a 2nd site lethal/sterile hit mutation. Presence of 2nd site lethal/sterile mutations on a transgenic constructs usually does not affect the outcome of functional studies as long as these transgenes are used in a heterozygous state (see **Figure 3**). **(B)** Generation of T2A-GAL4 lines through microinjection and crosses. Crossing scheme to convert a 2nd chromosome MiMIC insertion into a T2A-GAL4 element is shown here as an example. By microinjecting an expression vector for ϕC31 integrase and a RMCE vector for T2A-GAL4 (pBS-KS-attB2-SA-T2A-Gal4-Hsp70, must select appropriate reading frame for the MiMIC of interest. See the following papers for details[57,59]) into embryos carrying a MiMIC in a coding intron in gene of interest, one can convert the original MiMIC into a T2A-GAL4 line. See **Figure 2A** for a schematic diagram of the RMCE conversion. The conversion event can be selected by screening against the *y*$^+$ marker in the original MiMIC cassette[60]. Since RMCE can happen in two directions, only 50% of the successful conversion event will lead to successful production of GAL4, which can be detected by a UAS-GFP reporter transgene in the next generation. The final stable stock can be kept as homozygotes or as a balanced stock if the LOF of the gene is lethal/sterile.

Figure 2: Conversion of MiMIC elements into T2A-GAL lines via RMCE. **(A)** ϕC31 integrase facilitates the recombination between the two *attP* sites in the fly **(A-top)** and the two *attB* sites flanking a T2A-GAL4 cassette shown as a circular vector **(A-bottom)**. **(B)** Successful RMCE event leads to a loss of a selectable marker (*yellow+*), and insertion of the T2A-GAL4 cassette in the same orientation of the gene of interest. Since the RMCE event can happen in two orientations, only 50% of the RMCE reaction will give a desired product. RMCE product inserted in the opposite orientation will not function as a gene-trap allele and will not express GAL4. The directionality of the construct must be confirmed via Sanger sequencing. **(C)** Transcription **(C-top)** and translation **(C-bottom)** of the gene of interest leads to generation of a truncated mRNA and protein due to the polyA signal that present at the 3' end of the T2A-GAL4 cassette. The T2A is a ribosome skipping signal, which allows the ribosome to halt and reinitiate translation after this signal. This is used to generate a GAL4 element that is not covalently attached to the truncated gene product of interest. The GAL4 will enter the nucleus and will facilitate the transcription of transgenes that are under the control of UAS elements. UAS-GFP can be used as a gene expression reporter, and

UAS-human cDNA can be used for rescue experiments via gene 'humanization'. **(D)** Example of a T2A-GAL4 element in *bi* driving expression of UAS-GFP shown on the top. This expression pattern resembles a previously generated enhancer trap line for the same gene ($bi^{omb\text{-}GAL4}$) shown on the bottom. **(E)** Comparison of T2A-GAL4 allele of *bi* with previously reported LOF *bi* alleles. This figure has been adopted and modified from [57,87].

Figure 3: Functional Analysis of human variants using rescue-based (left) and over-expression (right) studies. **(A-left panel)**. The function of *EBF3* variants was assessed with a rescue-based analysis of the fly *knot (kn)* LOF allele focusing on lethality/viability. **(A-right panel)** The function of variants in *TBX2* was assessed by performing over-expression of human *TBX2* transgenes in wild-type flies, focusing on lethality/viability as well as eye morphology and electrophysiology phenotypes (see **Figure 4**). **(B)** Crossing schemes to obtain the flies that would be tested in the functional studies. One should always use a neutral UAS element (e.g. *UAS-lacZ*, *UAS-GFP*) as a control experiment. **(C)** Representative results from functional studies of $EBF3^{p.R163Q}$ and $TBX2^{p.R20Q}$ variants, respectively, along with appropriate control experiments that are necessary to interpret the results. Both the rescue-based analysis and over-expression studies reveal that the variants behave as amorphic or hypomorphic alleles. The lethality/viability data shown here are based on the experimental data presented in [15,87].

Figure 4: Functional analysis of a rare missense variant in human *TBX2* based on eye morphology and electroretinogram in *Drosophila*. **(A)** A schematic image showing the typical placement of recording and reference electrodes on the fly eye along with a representative electroretinogram recording with four major components (on-transient, depolarization, off-transient, repolarization). **(B)** *TBX2* variant (p.R20Q) functions as a partial LOF allele based on over-expression studies in the fly eye using GAL4 drivers specific to the visual system (*ey-GAL4* and *Rh1-GAL4*) showed that the reference TBX2 caused a strong morphological and electrophysiological phenotype compared to the variant protein. **(B-top panels)** A severe reduction in eye size is seen upon over-expression of $UAS\text{-}TBX2^{+}$ with *ey-GAL4*. $UAS\text{-}TBX2^{p.R20Q}$ driven with *ey-GAL4* also causes a smaller eye but the phenotype is much milder. **(B-bottom panels)** When $UAS\text{-}TBX2^{+}$ is expressed in core R1-R6 photoreceptors using Rh1-GAL4, there is a loss of the on-transient and off-transient, reduced depolarization, and a large abnormal prolonged depolarization after potential (PDA) phenotype that is not seen in control flies. These phenotypes are not as severe when UAS-$TBX2^{p.R20Q}$ is expressed using the same *Rh1-GAL4*. This figure has been adopted and modified from [69,87].

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Fig1

Click here to download Figure Figure 1.tif

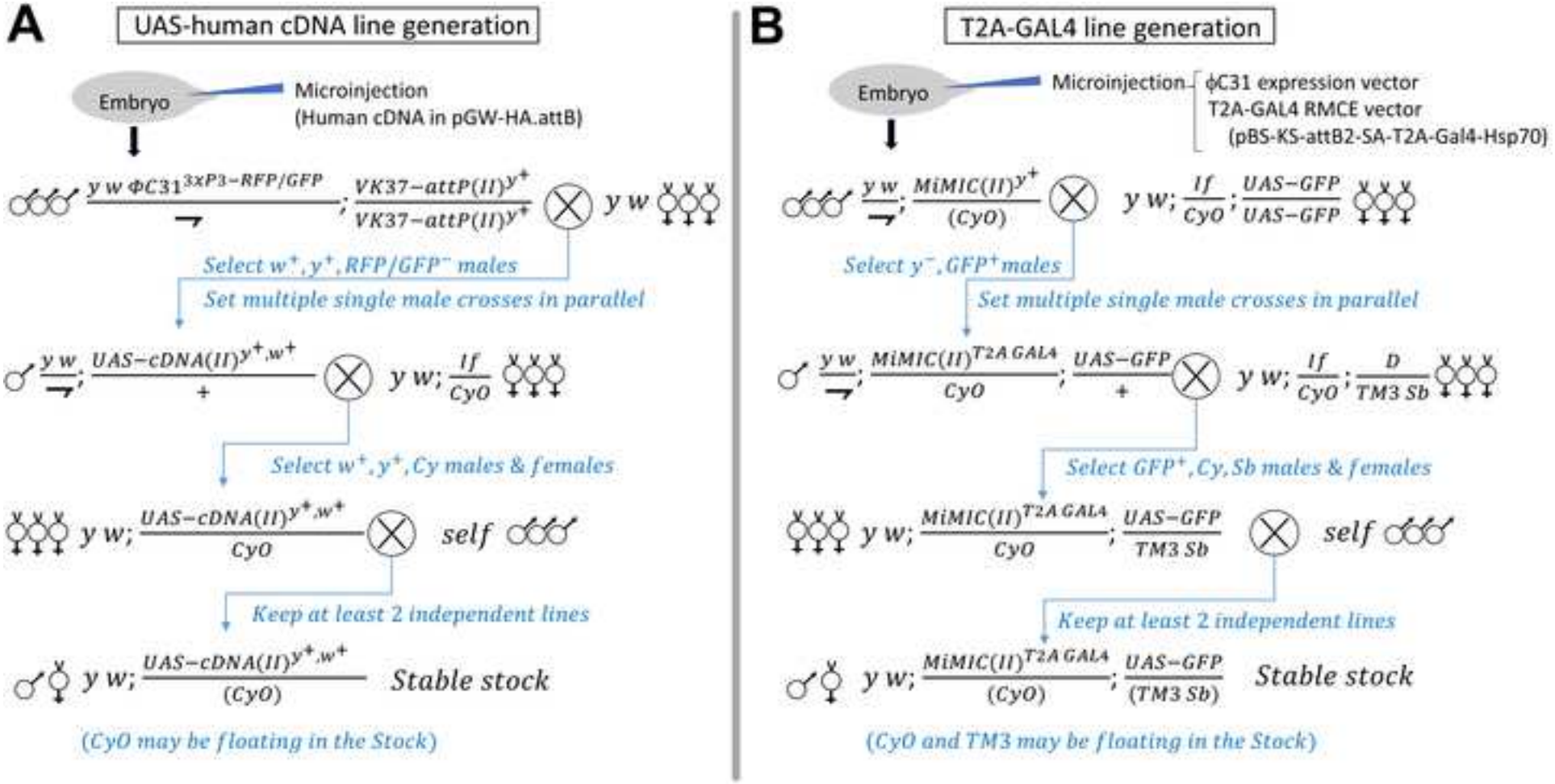

Fig2

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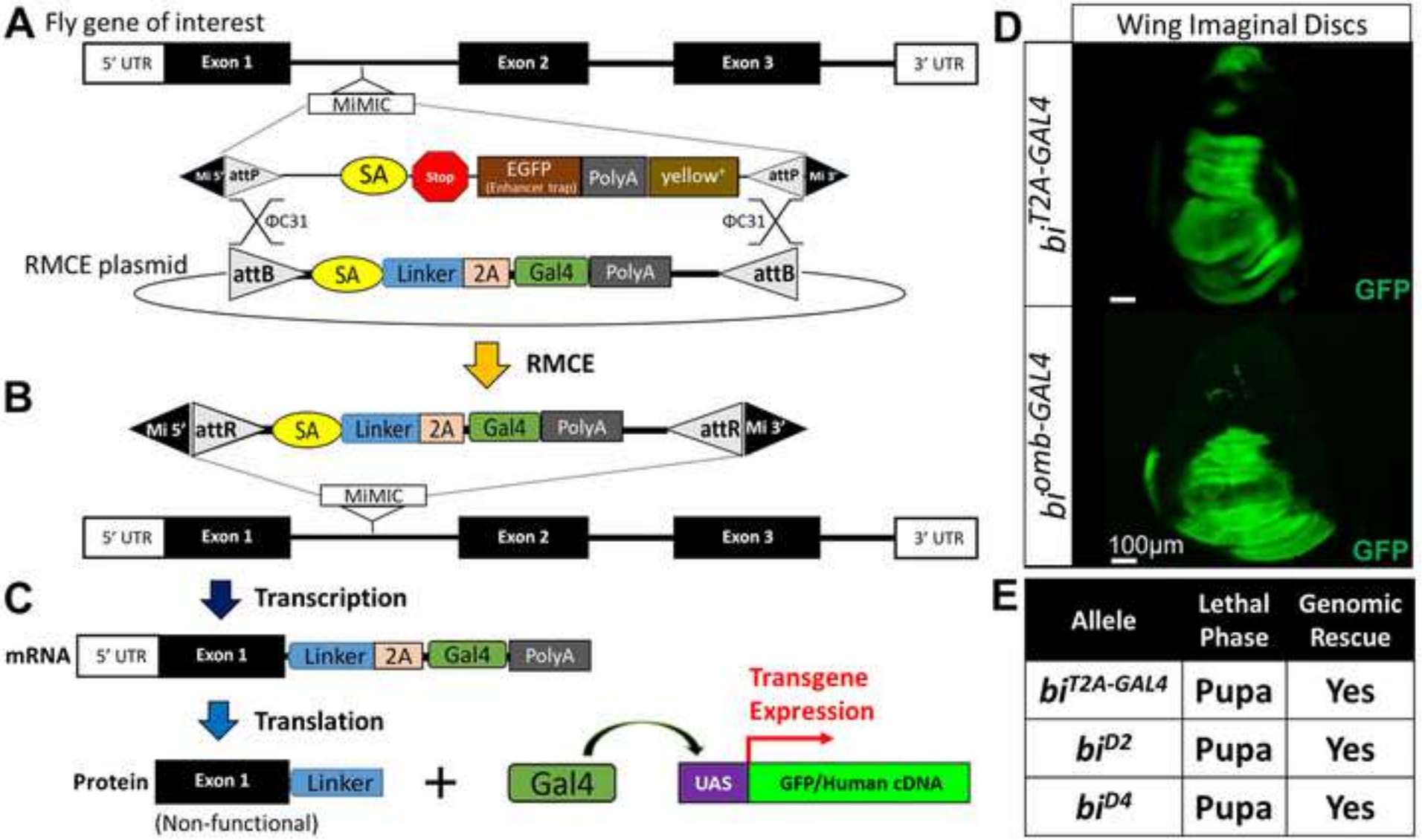

Allele	Lethal Phase	Genomic Rescue
$bi^{T2A\text{-}GAL4}$	Pupa	Yes
bi^{D2}	Pupa	Yes
bi^{D4}	Pupa	Yes

Fig3

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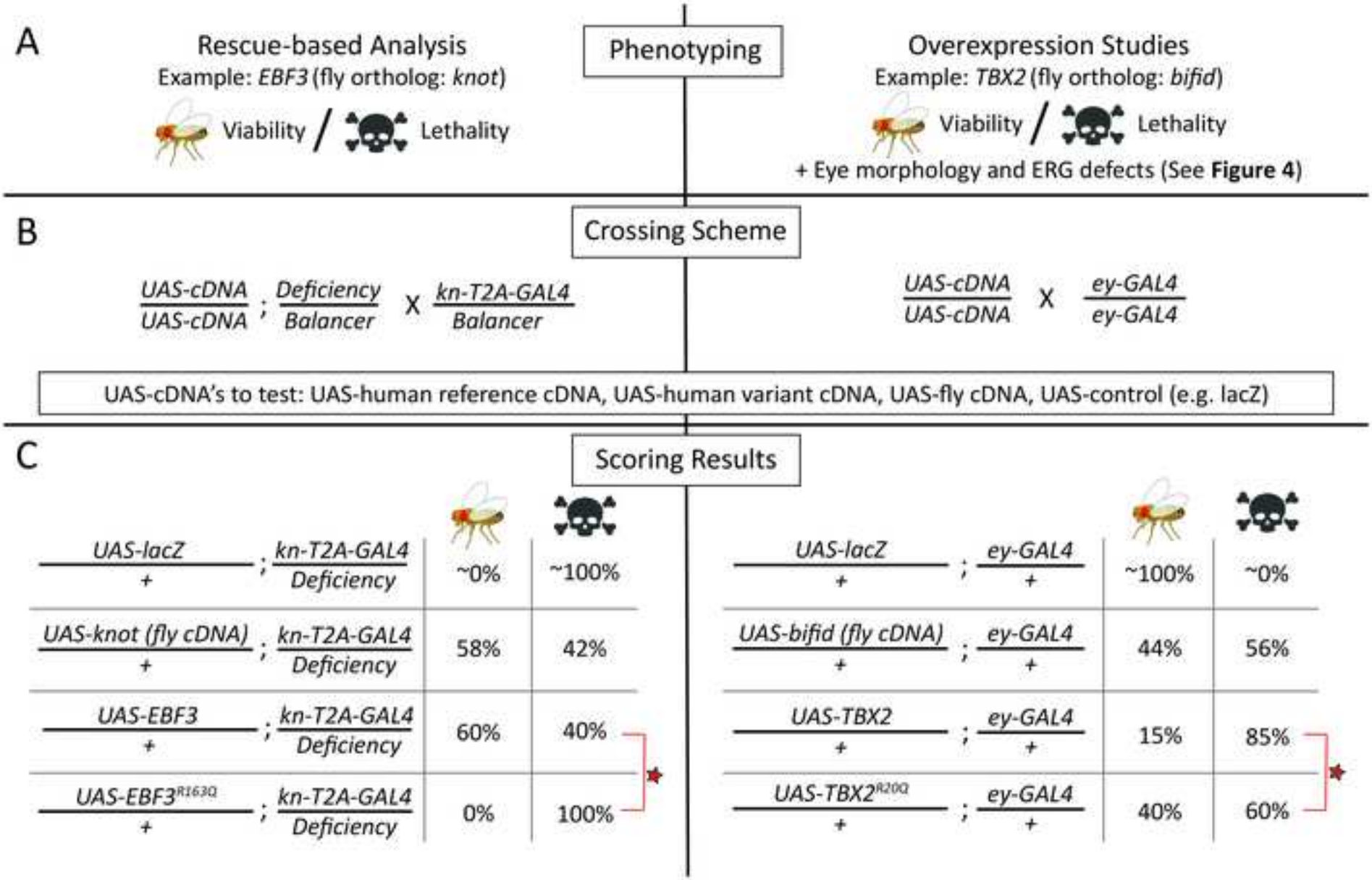

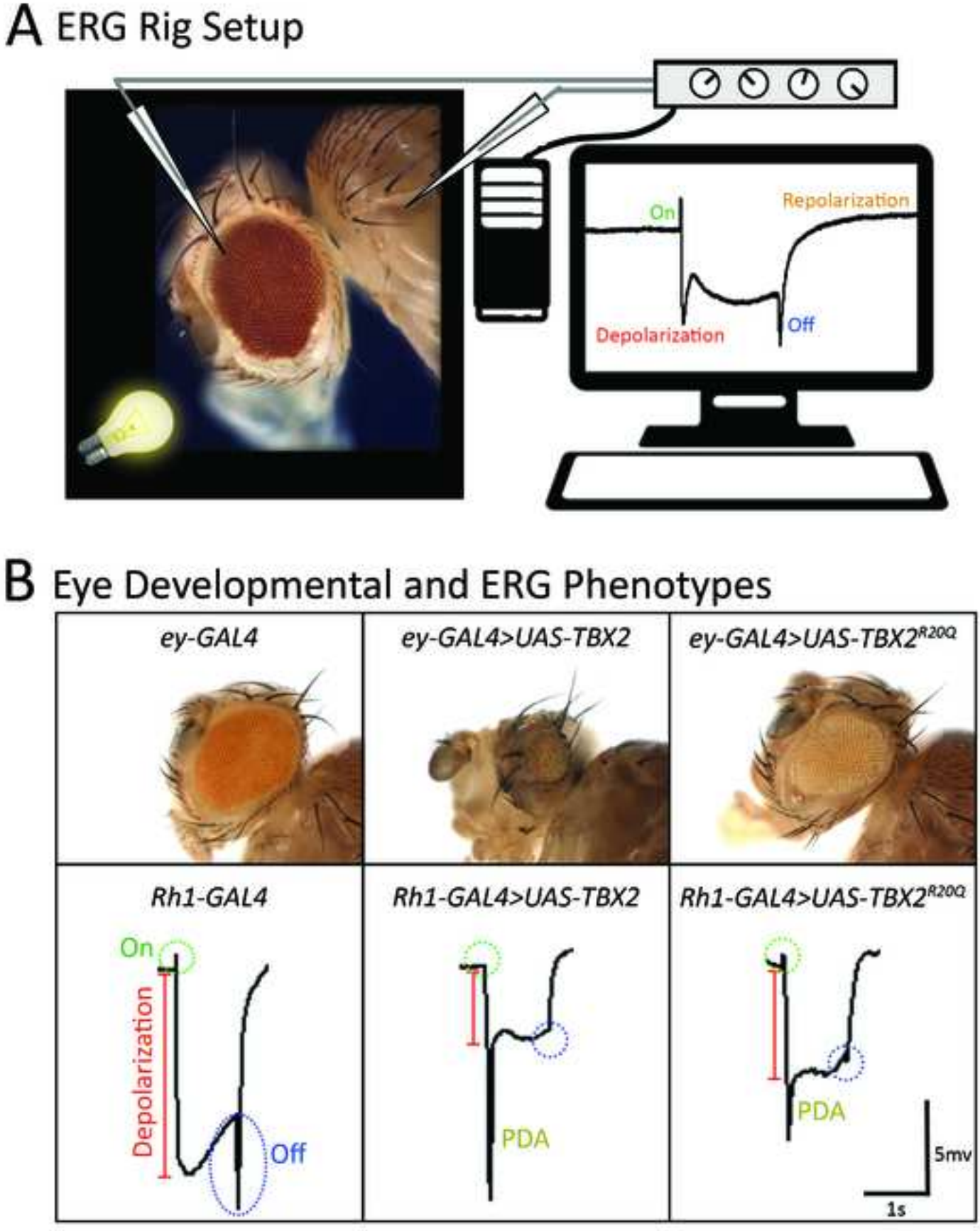

Table of Materials:

Drosophila Stocks for UAS-human cDNA transgenesis	
VK33 (3rd chromosome) injection line	BDSC
VK37 (2nd chromosome injection) line	BDSC
Plasmid DNA	
pDONR221	Thermo Fisher
pGW-HA. attB	Gift from Drs. Johannes Bischof and Konrad Basler (Bischof et al., 2013 PNAS)
Molecular biology kits and reagents	
Q5 Polymerase kit	NEB
BP Clonase kit	Thermo Fisher
LR Clonase II Enzyme kit	Thermo Fisher
PureLink Gel Extraction Kit	Thermo Fisher
Quick Change II Mutagenesis kit	Agilent
Agarose (molecular biology grade)	Sigma-Aldrich
QIAprep Spin Miniprep Kit	Qiagen
DH5α	Thermo Fisher
Electroretinogram Rig related equipment	
ISO-DAM Isolated Biologic Amplifier	LabX
Square Pulse Stimulator	Astro-Med
Axon pCLAMP 10 Data Software Package	Molecular Devices

#24871
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#12536-017
#M0491
#11789020
#11791100
#K210012
#200523
#A2790
#27104
#18265017
#R150358
#S48
N/A

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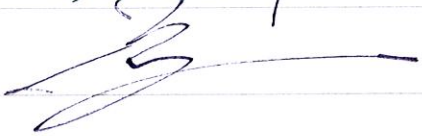
Signature: 

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