Studying the phenotypic variability of neurodevelopmental disorders with Drosophila melanogaster

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Abstract

The recurrent 520-kb 16p12.1 microdeletion, a rare copy-number variant significantly associated with severe developmental delay has been shown to exhibit wide phenotypic variability. A two-hit model has been proposed as an explanation for this phenotypic heterogeneity in patients with the 16p12.1 microdeletion and potentially other variably expressive neurodevelopmental disorders. According to this model, the 16p12.1 microdeletion would render an individual vulnerable to the manifestation of neuropsychiatric disorders, and the co-occurrence of secondary variants will lead to the manifestation of a more severe phenotype. To further understand how secondary variants push the sensitized genetic background towards the threshold for neurodevelopmental disease, we studied pairwise phenotypes of 16p12.1 genes with secondary variants in fruit flies. *Drosophila melanogaster* is a well-studied model organism for human genetic diseases, with 75% of human disease genes having an ortholog in flies. We selected twelve genes found mutated in 26 families carrying the 16p12.1 microdeletion by whole-exome sequencing, which are also associated with developmental disease. Eye-specific pair-wise knockdown of 16p12.1 orthologs and secondary variants was achieved to systematically examine the two-hit combinations against one-hit controls and the phenotypes were quantitatively measured using Flynotyper. Analysis of these results indicates additive and epistatic effects from the combination of the 16p12.1 microdeletion with other neurodevelopmental disease associated genes.

Introduction

A wide range of phenotypic severity characterizes many neurodevelopmental disorders, as observed with the 16p12.1 microdeletion resulting in the loss of 7 genes: *UQCRC2, PDZD9, C16ORF52, VWA3A, EEF2K, POLR3E, and CDR2*.[1] For example, children with the 16p12.1 microdeletion show significant phenotypic severity such as severe developmental delay, learning disabilities and craniofacial abnormalities.[1] However, parents carrying this same microdeletion show significantly less severe phenotypes including neuropsychiatric disease, learning disability and an increased risk for schizophrenia or depression.[1,2] The recently proposed two-hit model shows the presence of additional disease causing mutations in conjunction with 16p12.1 may explain some phenotypic variation, as the additional mutations surpass the tolerance threshold of

an individual.[1,2] Additionally, it has been shown that variation in genetic background of an individual has a significant impact on phenotypic severity resulting from a disease-causing mutation.[3] Therefore, we aimed to study the impact of variation in genetic background in conjunction with the 16p12.1 microdeletion in explaining phenotypic variability.

Drosophila melanogaster has been shown as an effective model organism for human disease, as it contains orthologs to 75% of human disease-associated genes.[4] Furthermore, the extensive knowledge of developmental processes and cell signaling of *Drosophila* provides an optimal environment for studying gene interactions within the context of a whole organism.[5,6] Likewise, through the use of tissue specific drivers, dispensable organs, such as the eye, can be utilized as experimental systems for genetic screening. Additionally, due to two-thirds of the *Drosophila* genome playing a vital role in eye development, phenotypic observations due to gene modifications can be translated to other tissues within the fly. The *Drosophila* eye also provides an ideal experimental system as it has a highly organized structure and thus defects in development result in readily observable phenotypes.[4,7] Computational methods can then be utilized to provide quantitative analysis of the resultant phenotypic severity.[4] Lastly, the *Drosophila* nervous system is highly similar to higher-level vertebrates enabling the impacts of orthologous genetic modifications of human neurodevelopmental disease-associated genes to be accurately translated to humans.[7]

High-throughput genome sequencing of 33 individuals carrying the 16p12.1 microdeletion was used to determine candidate genes potentially acting in concert with the microdeletion to result in phenotypic variation.[8] Functional genomic experiments using RNAi knockdowns in *Drosophila melanogaster* were then utilized for the fly orthologs of the 16p12.1 microdeletion conserved genes *UQCRC2, C16ORF52*, *POLR3E,* and *CDR2*. Additionally, the GAL4-UAS system was used to knockdown these gene orthologs in the eye to enable observation of gene interactions within a whole animal model. These knockdowns were then combined pairwise with knockdowns of 12 candidate genes (*SETD5, LAMC3, DMD*, *ARID1B, DST, NALCN, PDE11A, USP45, CAPN9, DNAH10, CACNA1A,* and *PYGM*) to systematically analyze and determine the level of gene interaction and subsequent phenotypic severity. Phenotypic severity of the fly eye was determined quantitatively using Flynotyper to provide a possible explanation for the phenotypic variation in individuals carrying the 16p12.1 microdeletion.[4] Analysis of these pairwise combinations of the 16p12.1 deletion as the first-hit and candidate gene as the second-hit indicated two-hit combinations were largely additive or suppressive. However, some combinations indicated significant enhancement of the two-hit phenotype compared to the first-hit and second-hit individually as seen with *C16ORFf52* in combination with *SETD5*.

Methods

Drosophila Stocks

Knockdowns for specific genes to produce the two-hits and one-hits were achieved through the UAS-GAL4 system and RNAi with w;GMR-GAL4 and UAS-RNAi transgenic lines. Within this study the following RNAi fly stocks were utilized from the Bloomington Drosophila Stock Center: UAS-*upset*^{RNAi} (BDSC# 51447, 61266), UAS-*lanb2*^{RNAi} (BDSC# 62002), UAS-*dys*^{RNAi} (BDSC# 55641, 31553), UAS-*osa*^{RNAi} (BDSC# 35447, 38285, 31266), UAS-*shot*^{RNAi} (BDSC# 41858, 64041, 28336), UAS-*na*^{RNAi} (BDSC# 26704), UAS-*pde6*^{RNAi} (BDSC# 35743, 25828),

UAS-*usp16-45*^{RNAi} (BDSC# 22338, 11326), UAS-*astc-r2*^{RNAi} (BDSC# 36888, 25940), UAS-*calpa*^{RNAi} (BDSC# 29455), UAS-*dhc98d*^{RNAi} (BDSC# 23611), UAS-*cac*^{RNAi} (BDSC# 965), UAS-*glyp*^{RNAi} (BDSC# 10692), UAS-*dmyc*^{RNAi} (BDSC# 9674, 9675, 25783, 43962, 64769). Fly stocks for GMR-Gal4-*cen*^{RNAi} (VDRC# 33444), GMR-Gal4-*CG4169*^{RNAi} (VDRC# 26404), GMR-Gal4-*sin*^{RNAi} (VDRC# 51696), GMR-Gal4-*CG14182*^{RNAi} (VDRC# 5370) and w;dcadGMR-Gal4/cyo were obtained from the Vienna Drosophila Resource Center.[9] All fly stocks were cultured under conventional conditions of cornmeal/sucrose/dextrose/yeast medium at 25°C. At least two RNAi lines were utilized per candidate gene when possible to improve confidence in interaction classification of the two-hit combinations.

Eye imaging using bright-field microscopy

1 to 4 day-old flies carrying the eye-specific driver GMR-Gal4 and a hairpin sequence complementary to the target gene under the expression of UAS (UAS-RNAi) were cultivated at 30°C were frozen at -80°C. Once immobilized the flies were mounted on Blu Tack (Bostik Inc, Wauwatosa, WI) for imaging with bright-field microscopy. These adult fly eyes were then imaged using a Semimotorized Olympus BX53 microscope with a 20x objective and 0.5x magnification C-mount camera (Olympus, Tokyo, Japan). CellSens Dimension software (Olympus Optical) was used to capture these images, which were then stacked with Zerene Stacker (Zerene Systems, Richland, WA).[4] Flynotyper software was used to quantitatively assign phenotypic scores for the first-hit, second-hit, and two-hit eye phenotypes.[4] Overall, phenotypic score correlates the ommatidial disorderliness and subsequently a more severe phenotype is represented by a higher phenotypic score.

Interaction Classification

Flynotyper generated phenotypic scores were plotted into GraphPrad Prism (GraphPad Software, Inc.) as min to max box and whiskers. Two-tailed Mann-Whitney statistical analysis was performed using MiniTab software (MiniTab, Inc.) with statistical significance as $p \leq 0.05$. Flynotyper results were obtained from an average of 10-15 fly eyes.

Results

Targeted RNAi knockdown within the eye systematically produced flies with phenotypes for the first-hit, second-hit and two-hit combinations. These combinations comprised knockdown of the 16p12.1 orthologs as the first-hit (A) and knockdown of candidate genes (B) as the second-hit. The double knockdown comprised pairwise knockdown of both the 16p12.1 and candidate gene orthologs (A+B). Comparison of the phenotypic severities of the first-hit and second-hit to the two-hit phenotype revealed three general cases: addition, suppression and enhancement. Addition was characterized when the phenotypic score of the combination of both genes (A+B) was approximately the sum of the scores of the each of them individually (A and B) (Figure 1A). Suppression was distinguished when the phenotype of the double knockdown of A+B was milder (evidenced by a lower Flynotyper score) compared to the first-hit and second-hit individually (Figure 1B). Then, a two-hit phenotypic score that was notably higher than the sum of the one-hit and second-hit scores in multiple RNAi lines for the same gene indicated enhancement (Figure 1C). Again, two or more RNAi lines were used, if available, to produce the

two-hit phenotypes to improve confidence in determining the sensitivity of the 16p12.1 genes to variation in genetic background.

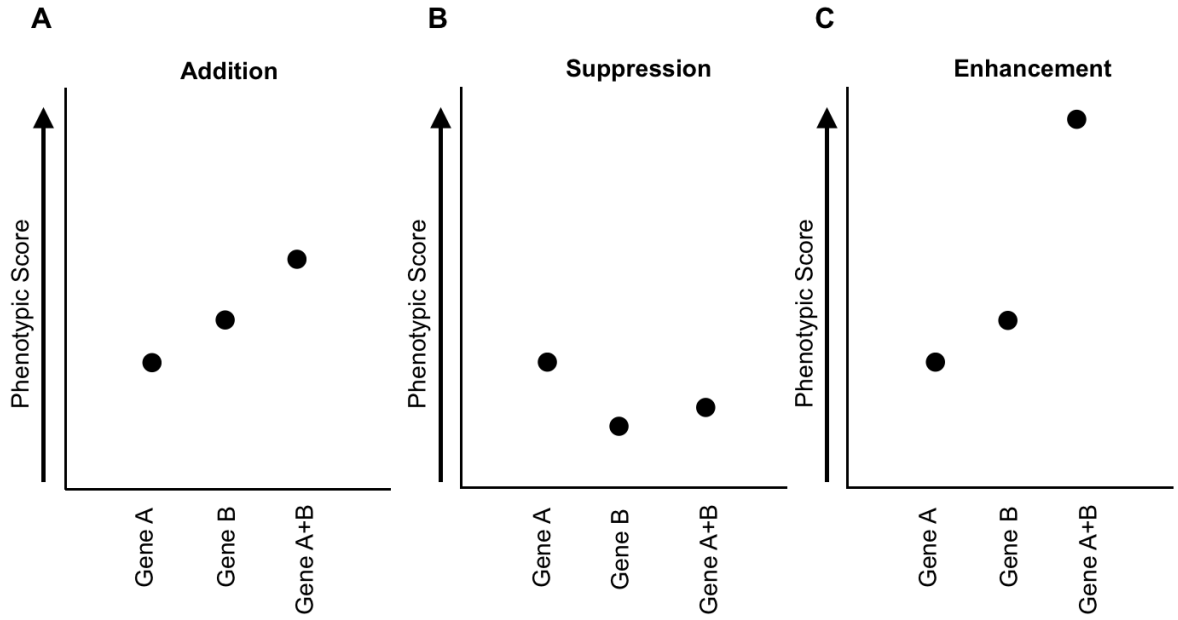

Figure 1: Characterization of observed gene interactions. Representation of characterization criteria used to identify observed interactions manifesting the double knockdown phenotypes as addition (A), suppression (B), or enhancement (C). Gene A is representative of the first-hit comprising 16p12.1 ortholog knockdown and gene B represents the candidate gene ortholog knockdown as the second-hit. Gene A+B represents the double knockdown phenotype resulting from simultaneous knockdown of gene A and gene B.

The majority of pairwise combinations between the 16p12.1 and candidate gene orthologs showed an additive or suppressive effect (Figure 2). Variation in the amount of addition and suppression was also observed within these combinations. Additionally, phenotypic scores for the two-hits were often approximately equal to the second-hit phenotypic score as a result of the second-hit conferring a severe phenotype. For example, *LAMC3* orthologous knockdown produced flies that had few to no ommatidia when it was the only hit to the genome. As a result, the combination of the mild phenotype from the 16p12.1 othologous knockdown was not able to further increase the ommatidial disorder. A strong indication of enhancement was observed with the double knockdown of *C16ORF52* and *SETD5* orthologs (Figure 3). Additionally, double knockdown of *POLR3E* and *USP45* shows some enhancement. However, because only data for one RNAi line for *USP45* was available additional testing is required to conclude this interaction.

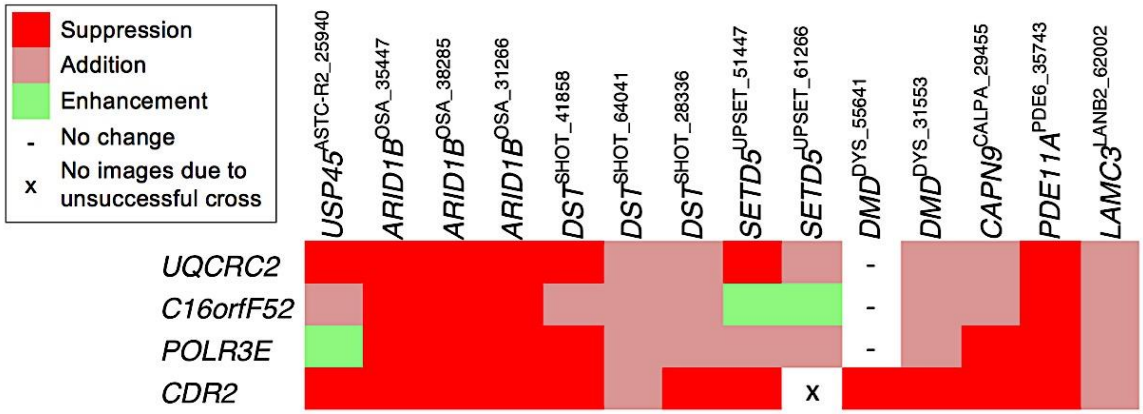

Figure 2: Pairwise knockdown of 16p12.1 and candidate gene orthologs. Combinations of pairwise knockdowns tested and characterization of their interactions. Enhancement of phenotype severity was only observed in the two-hit combinations for *C16orf52*/*SETD5* and *POLR3E*/*USP45*.

Discussion

These results support the two-hit model that the 16p12.1 genes are sensitive to changes in the genetic background. Furthermore, the observation of suppression, addition and enhancement further support genetic background playing a crucial role in generating the phenotypic heterogeneity observed within individuals carrying the 16p12.1 microdeletion. Thus, the specific interactions between these genes remain to be examined to determine if genes are acting within similar pathways or in an epistatic manner. For example, *POLR3E* encodes RNA polymerase III subunit E (http://www.genecards.org/cgi-bin/carddisp.pl?gene=POLR3E) and is required in catalyzing transcription of DNA to RNA. Thus, it maintains an important role within gene expression. Furthermore, *USP45*, which encodes ubiquitin specific peptidase 45, is responsible for maintaining the activity of ERCC1-XPF DNA repair activity (http://www.genecards.org/cgi-bin/carddisp.pl?gene=USP45). Additionally, loss of ERCC1-XPF function has also been shown to have severe impacts on development (Al-Minawi et al. 2008). Thus, further characterization into the pathways of these proteins may provide insight into the manifestation of the more severe phenotypes observed in individuals carrying the 16p12.1 microdeletion. However, *C16ORF52* remains an uncharacterized gene. Nevertheless, an indication of enhancement with loss of *SETD5* provides an opportunity to further characterize the function of the *C16ORF52* gene. While *SETD5* also remains to be characterized, research indicates that it has sequence similarity to other characterized SET domain proteins such as *SETD7*, known to encode the histone-lysine methyltransferase SET7/9 (http://www.uniprot.org/uniprot/Q8WTS6; Marmorstein 2003). Thus, *C16ORF52* may be impacted in a primary or secondary way by changes in chromatin remodeling and subsequent gene regulation potentially related to loss of *SETD5* activity.

Furthermore, because these pairwise combinations have only been examined in respect to *Drosophila* eye development, impacts of these combinations should be examined further to ensure their accurate translation to nervous system development. Therefore, these observations will be integrated with gene expression data (RNA-seq) available from *Drosophila* brains with decreased expression of *UQCRC2*, *C16ORF52*, *POLR3E*, and CDR2.

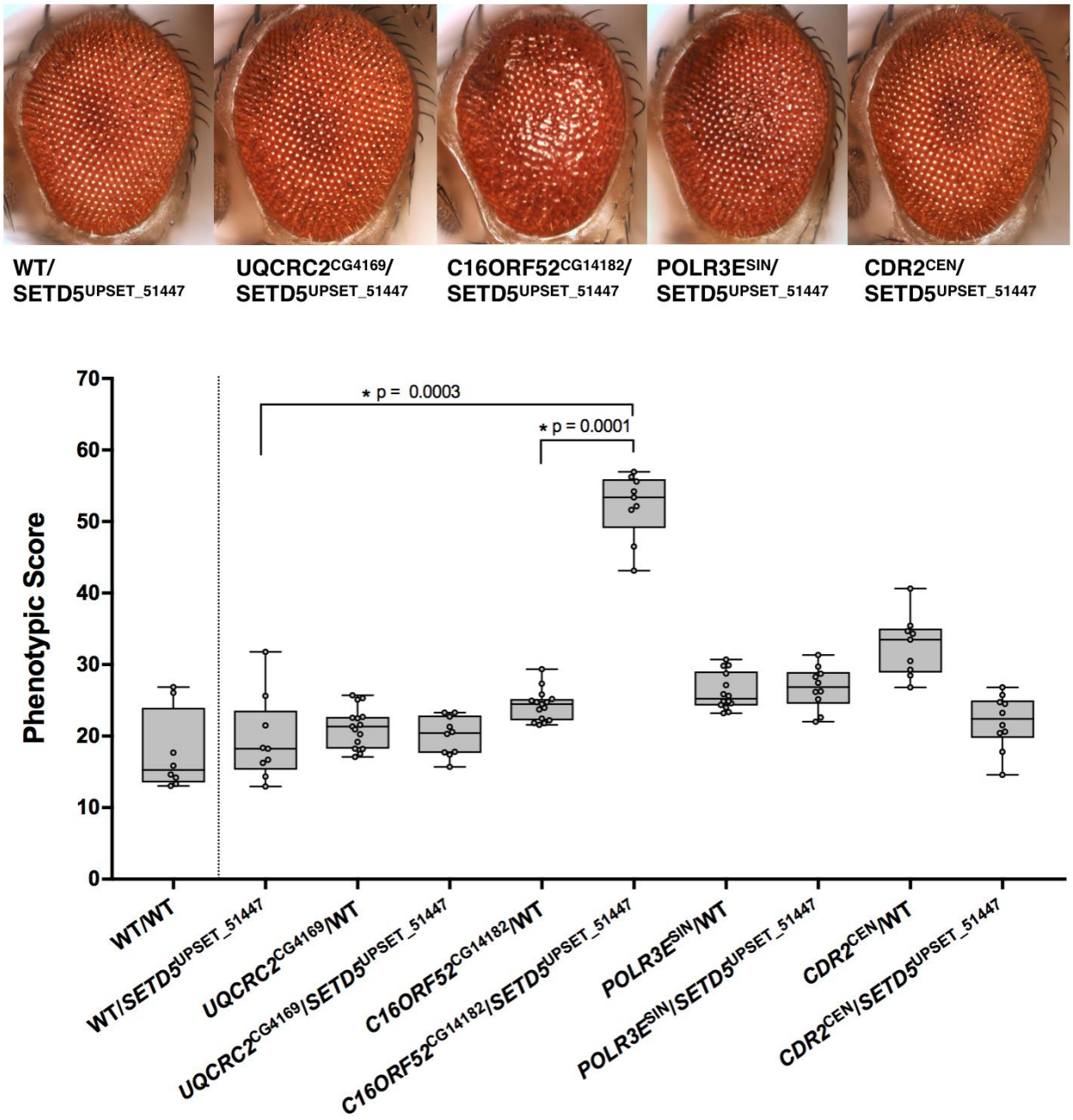

Figure 3: *C16orf52*/*SETD5* enhancement of phenotypic severity. Significant enhancement of phenotypic score is observed only for the ortholog of *C16orf52* in combination with *SETD5*. Two-tailed Mann-Whitney test confirmed a significant difference between the double knockdown and the first and second-hit phenotypes. This enhancement was also seen in the second RNAi line for *SETD5* (UPSET_61266: first-hit to two-hit, p=0.0000; second-hit to two-hit, p=0.0004). The results shown for *CDR2* may suggest a suppression effect with *SETD5*. However, results from a secondary RNAi line are necessary to conclude an interaction among these genes. The 16p12.1 orthologs *UQCRC2* and *POLR3E* did not show a significant difference of the eye phenotype compared to the one-hit.

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