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Dissecting the complexity of CNV pathogenicity: insights from *Drosophila* and zebrafish models

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Abstract

Genetic architecture predisposes regions of the human genome to copy-number variants, which confer substantial disease risk, most prominently towards neurodevelopmental disorders. These variants typically contain multiple genes and are often associated with extensive pleiotropy and variable phenotypic expressivity. Despite the expansion of the fidelity of CNV detection, and the study of such lesions at the population level, understanding causal mechanisms for CNV phenotypes will require biological testing of constituent genes and their interactions. In this regard, model systems amenable to high-throughput phenotypic analysis of dosage-sensitive genes (and combinations thereof) are beginning to offer improved granularity of CNV-driven pathology. Here, we review the utility of *Drosophila* and zebrafish models for pathogenic CNV regions, highlight the advances made in discovery of single gene drivers and genetic interactions that determine specific CNV phenotypes, and argue for their validity in dissecting conserved developmental mechanisms associated with CNVs.

Introduction

Copy-number variants (CNVs) are a major contributor to complex genetic disorders, accounting for about 25% of individuals with autism, intellectual disability/developmental delay (ID/DD), epilepsy, and schizophrenia [1–3]. The most frequently-studied CNVs, such as duplications and deletions at 16p11.2, 16p12.1, 3q29, and 1q21.1, are associated not only with a diverse set of clinical features, but also with variable expressivity [3]. Dissection of the breakpoints of CNVs, evaluation of genes perturbed within CNVs in patient populations,

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and modeling genes in model organisms is beginning to unravel the genetic basis as well as causal functional mechanisms for these disorders, the lessons of which are likely useful across human genetics. In this review, we will focus on a subset of well-studied CNVs and discuss how animal modeling in particular can contribute to our understanding of pleiotropy and epistasis.

The phenotypic and genotypic landscape of CNVs—current challenges.

While CNVs pose a challenge for detection and interpretation in the context of disease pathology, they also represent an opportunity to study two major issues in medical genetics: pleiotropy and variable expressivity. In some cases, single-gene mutations within the CNV, such as deleterious mutations in *RAI1* located within 17p11.2 deletion in Smith-Magenis syndrome (SMS), account for the majority of the observed phenotype [4–6]. However, these instances are outliers, especially when a CNV is associated with pleiotropic features. For example, the 16p11.2 deletion is associated with ID/DD [7–9], cardiac disease [10], epilepsy [11], and obesity [12], and it accounts for 1% of sporadic autism [13,14]. Identifying the genetic basis of phenotypes associated with these variably expressive CNVs has been challenging for several reasons. *First*, CNVs associated with variable expressivity are not amenable to causal gene discovery using atypical deletions or chromosomal translocations [3,15]. *Second*, large-scale sequencing studies of affected individuals have not identified causal genes that solely explain the clinical features of the entire CNV. For example, while mutations in *TAOK2* within 16p11.2 have been identified in individuals with autism, their clinical features do not recapitulate the full range of phenotypes of 16p11.2 deletion carriers [16]. *Third*, mouse models of individual CNV genes do not recapitulate the phenotypes observed in rodent models of the entire CNV. For example, individuals genes within the 3q29 and 15q13.3 deletions, including *DLG1*, *PAK2*, *CHRNA7*, and *OTUD7A,* each showed developmental pathologies but did not account for the full range of phenotypes of the whole deletion [17–21]. *Fourth*, the biological mechanisms for pathogenicity of CNVs are relatively unknown. In fact, mechanisms for *RAI1* were not well described until recently, when studies found that *RAI1* acts in specific neuronal subtypes [22] and affects synaptic plasticity and scaling [23]. Overall, these findings suggest that variably expressive clinical features of CNVs are likely due to combinatorial effects of multiple candidate genes.

Understanding CNV pathogenicity using fly and zebrafish model systems.

Fly and zebrafish models allow for high-throughput evaluation of multiple individual homologs and complex genetic interactions, with low husbandry costs and availability of genetic tools to mimic alterations of CNV gene expression. Over 75% of human disease genes have homologs in *Drosophila melanogaster*, including many genes involved in conserved developmental and neuronal processes [24]. Moreover, tools such as the *UAS-GAL4* system and RNA interference allow for simultaneously modulating the expression of multiple genes, making *Drosophila* a powerful system to test genetic interactions in a tissue-specific manner. For example, the fly eye has been used to perform high-throughput analysis of genes involved in Rett syndrome, spinocerebellar ataxia, and intellectual disability disorders [25–27]. Furthermore, *Drosophila* models have been used for assessing multiple developmental, neurological, and behavioral functions, including cardiac physiology [28], dendritic and synaptic architecture [29–31], learning and memory [32–34], and sleep and

circadian function [•35,36]. Importantly, this model organism has allowed the query of both single genes and combinations thereof, exemplified by Grossman and colleagues who identified cooperative interactions between candidate Down syndrome genes, *DSCAM* and *COL6A2*, towards congenital heart defects [28]. Similarly, *Drosophila* models of 22q11.2 deletion identified *LZTR1* as a candidate gene that affects sleep activity through its interactions with *NF1* and modulating GABA signaling [•35].

Similar to *Drosophila*, zebrafish have emerged as valuable model for functional genomic studies, with a vertebrate body plan and conserved patterns of cell movement, differentiation, and organogenesis. Zebrafish have orthologs for ~ 70% of human genes, a number that rises to 84% when considering genes associated with human clinical traits [37]. Body transparency during early development and completion of organogenesis by five days post fertilization allows for the direct observation of developmental processes in real time. In addition, the ability to create stable transgenic lines with tissue specific expression advanced the utility of zebrafish for translational studies [38]. Although imperfect and subject to the need for extensive validations to ensure reagent specificity, transient suppression and overexpression systems have proven particularly useful in CNV dissection, allowing for scaled assessment of most genes within a CNV – a feat difficult to accomplish with stable mutants in any species. Systematic analysis of CNV genes using transient dosage perturbation during development has enabled unraveling of distinct pathologies, including neuroanatomical [39], behavioral [26, •35,40,41], and comorbid craniofacial defects [•42]; as well as manifestations in other vital organs [28,43].

Insights into mechanisms: ‘minimal overlap’ and genetic interactions.

Given the challenges of mapping individual genes to specific clinical features of variably-expressive CNVs, recent studies have used animal models to evaluate the role of conserved homologs of each CNV gene towards quantitative neuronal phenotypes. Systematic evaluation of hundreds of pairwise interactions using *Drosophila* have been instrumental in understanding the genetic etiology of CNV regions, including 16p11.2 [•44], 3q29 [45], and 22q11.2 [•46,47]. Using quantitative neuronal and cellular assays, several studies have demonstrated that interactions between CNV homologs can act as either suppressors or synergistic or additive enhancers of phenotypes observed for individual homologs [48]. For example, Grice and colleagues showed that genes within CNVs identified from individuals with autism, including *DLG1*, *PAK2*, and *TBX1*, synergistically lead to synaptic and sleep behavior defects [49]. Similarly, *Drosophila* models of *SLC25A1* and *MRPL40*, genes located within 22q11.2 deletion, and *SLC25A4*, a transcriptional target for *SLC25A1*, interact toward defects in synaptic development, plasticity, and function [45,•46]. In addition to identifying distinct interaction patterns for genes across different CNV regions, such studies have also uncovered putative biological mechanisms affected by these interactions. For example, homologs of *NCBP2*, on 3q29, exacerbated the neurodevelopmental and axon targeting defects of 14/16 other 3q29 homologs, suggesting that it potentially acts as a key modifier within the deletion [45]. Apoptosis was identified as a likely cellular mechanism mediating these defects due to interaction of 3q29 homologs, which was further validated by rescue of the observed defects by the apoptosis inhibitor *DIAP1* [45]. Several candidate genes in CNV regions are likely involved in processes critical for early development,

as a screen of about 60 CNV homologs for defects during fly wing development found *NCBP2*, *POLR3E*, *PPP4C,* and other candidate genes that disrupted conserved signaling pathways, such as Wnt, Notch, and Hedgehog [50]. Thus, *Drosophila* studies have found that interaction patterns of CNV homologs differ by CNV region and are modulated by key neurodevelopmental mechanisms.

Similarly, zebrafish models have been leveraged to identify complex molecular mechanisms leading to structural malformations, such as neuroanatomical and renal defects. For instance, both deletions and duplications in 17p13.1 have been linked to global developmental delay, intellectual disability and microcephaly phenotypes in children [51–53]. Analysis of affected individuals with overlapping CNVs in 17p13.1 defined a critical interval of approximately 160 kbp containing nine genes. Transient overexpression of all nine human transcripts in zebrafish identified seven genes as contributors to head-size defects, with the majority of the genes (6/7) inducing microcephaly in the reciprocal suppression experiments [53]. The authors observed that most genes tested within 17p13.1 gave rise to a patient-relevant phenotype for both the deletion and duplication, and interaction studies performed by suppressing or overexpressing gene pairs using suboptimal doses identified defects that recapitulated or exceeded the effect of single genes. A similar paradigm also emerged from more recent studies of DiGeorge syndrome, caused by 22q11.2 deletion and characterized by variable expressivity of multiple features, including cardiac malformations, immune deficiency, and neurodevelopmental and urogenital defects [54–56]. Lopez-Rivera and colleagues performed genome-wide search in patients with congenital kidney anomalies without characteristic DiGeorge symptoms, and identified heterozygous deletions of a specific 370 kbp interval within 22q11.2 in about 1.1% of the patients [57]. Functional studies in zebrafish revealed that loss of function of *snap29*, *aifm3*, and *crkl* resulted in convolution defects of the proximal pronephros and overall shortening of the renal tubules [57]. Inactivation of *Crkl* in a mouse model further induced developmental defects similar to those observed in patients with congenital renal defects. These results suggested that renal defects associated with this CNV are driven primarily by *CRKL* and its interactions with other genes in the phenotype-specific critical interval [57]. In essence, the amalgam of clinical studies and the presence of multiple dosage-sensitive genes within these CNVs highlight the concept of ‘minimal overlap’, where each CNV is hallmarked by a penetrant trait driven either by a single gene or a small subset of dosage-sensitive genes, which are further modulated by complex genetic interactions.

Modeling the 16p11.2 CNV: causality and epistasis.

The concepts described above are best exemplified by a series of studies of the 16p11.2 CNV, a reciprocal lesion that has benefited from extensive genetic and animal modeling. In the 16p locus, five chromosomal breakpoints have been identified to be prone to recombination events, which give rise to different size deletions and duplications. The boundaries of the unstable region, defined by breakpoints 1 and 5 (BP1-BP5), is 1.7 Mbp and contains 64 genes. The two most frequent (and thus studied most intensely) subregions are the distal BP2-BP3 220 kbp region, containing 9 genes, and the proximal BP4-BP5 600 kbp region, containing 29 genes. Both CNVs are associated with neurodevelopmental defects and other comorbid features such as craniofacial, skeletal, and metabolic phenotypes.

The majority of animal work has focused on the proximal BP4-BP5 CNV and its involvement in neurodevelopmental defects [•44,58,•59]. Iyer and colleagues reduced expression of individual homologs of 16p11.2 genes in *Drosophila* and identified a range of developmental phenotypes, including early larval and pupal lethality for 7/14 homologs as well as robust wing defects for 12/14 homologs (Fig. 1) [•44]. Several of the genes, including *KCTD13*, *CORO1A*, *MAPK3*, and *ALDOA*, led to severe neuronal defects in motor function, neuromuscular junction morphology, axon targeting, and cellular defects (Fig. 1) [•44]. Furthermore, homologs of 16p11.2 genes, including *KCTD13, PPP4C, MAPK3, and DOC2A*, interacted with each other by enhancing or suppressing cell proliferation phenotypes in developing *Drosophila* neuronal tissues [•44]. Genetic interactions identified from *Drosophila* studies were further validated in the context of human brain-specific gene interaction networks (Fig. 2), with human 16p11.2 genes showing strong connectivity with each other in the network [•44,60]. In fact, connector genes in the 16p11.2 deletion network were enriched for cell proliferation functions, corroborating results from *Drosophila* and zebrafish functional assays [•44]. These studies also found additional interactions among CNV genes that did not have conserved homologs in *Drosophila*, such as *MAZ* and *MVP* within 16p11.2, and between homologs in different CNV regions, including an interaction between *TAOK2* in 16p11.2 and *TUFM* in distal 16p11.2 (Fig. 2).

Using zebrafish models, Blaker-Lee and colleagues explored the impact of 22 homologs of proximal 16p11.2 genes and found that a majority of the genes contributed to neuroanatomical (90% of genes), tail (~72% of genes), and touch response defects (~63%) (Fig. 3) [58]. A subsequent study identified *KCTD13* as a major driver for the neuroanatomical defects observed with both deletion and duplication of this region [•59]. While additional *KCTD13* cases [•59,61] and transcriptomic analysis of animal and cellular models further supported this finding [•44,62], follow-up studies have complicated the interpretation [63]. Further studies using pairwise interaction models did not find a major driver for ventricle morphology defects, but instead detected complex interactions between a subset of six loci, including *fam57a*, *kif22*, *asphd1*, *hirip3*, *kctd13,* and *sez6l2* (Fig. 3) [•64]. Similarly, systematic analyses of the distal 16p11.2 region showed that *LAT* overexpression was sufficient to cause neuroanatomical defects. In fact, genetic interactions between *LAT* and proximal 16p11.2 genes that contribute to microcephaly further exacerbated the observed phenotype (Fig. 3). Furthermore, chromatin conformation studies confirmed that genes within the proximal and distal regions physically align in 3D space [65], which potentially explains the increased severity of microcephaly in patients with deletions encompassing both regions [66]. In another study, Qiu and colleagues explored morphometric data from affected individuals along with mouse, rat, and zebrafish models to dissect the genetic drivers of these phenotypes. Morphometric features obtained from 3D facial imaging of CNV carriers revealed opposite effects between deletion and duplication carriers, while the genetic effects clustered to genomic regions corresponding to major processes of craniofacial development [•42]. Overexpression of *KCTD13* along with *MVP* and *MAPK3* in zebrafish resulted in significant decrease of the ceratohyal angle. In contrast, reciprocal loss of *mapk3* alone or in combination with *mvp* and *kctd13* resulted in increase of the ceratohyal angle [•42]. Finally, the mirror effects of 16p11.2

deletions and duplications were recapitulated in both mouse and rat models, supporting results from zebrafish studies. Thus, systematic analysis of *Drosophila* and zebrafish models unraveled the complexity of genetic interactions contributing to neurodevelopmental features often observed in patients with 16p11.2 CNVs. Several individual genes were sufficient to negatively affect major developmental processes, including cell proliferation and axon tracking, while genetic interactions of genes in both proximal and distal regions modulated the observed phenotype.

Challenges of using *Drosophila* and zebrafish models for studying CNVs.

Drosophila and zebrafish models allow for high-throughput evaluation of conserved homologs of genes within CNV regions and their interactions toward developmental phenotypes. The fact that findings from fly and zebrafish studies, such as complex interactions within the 16p11.2 deletion, show concordance with each other [•42, •44, •64] strengthens the utility of these models. At the same time, we must also highlight the limitations associated with studying CNV disorders using these approaches. For instance, model systems cannot recapitulate a subset of clinical and behavioral features of complex disorders observed in humans. In addition, the presence of multiple orthologs for a single human gene or a single ortholog for multiple human genes makes it challenging to properly address phenotypes observed due to single gene modulation, while overexpression experiments that mimic increased copy-number genomic events are an imperfect dosage proxy prone to false positives and negatives. Moreover, phenotypes due to disruption of individual genes can also be enhanced or suppressed by the genetic background of the model system, while gene interaction networks may be species-specific and may not recapitulate interactions in mammalian models or humans [67]. For example, *NCBP2* was not identified as a strong candidate in a mouse model of 3q29 deletion, suggesting species-specific effects [17]. Despite these challenges, *Drosophila* and zebrafish models provide a robust genetic tool that can complement mammalian models of the entire deletion, and can aid in fine-mapping the phenotypes observed in whole deletion models to individual homologs and their interactions. In a world in which CNV identification will continue to accelerate due to the ever-increasing abundance of genomic data from humans, such tools to methodically study the contribution of genes and their interactions as they pertain to causality and pleiotropy will continue to offer a level of resolution that bulk genomic studies will struggle to achieve.

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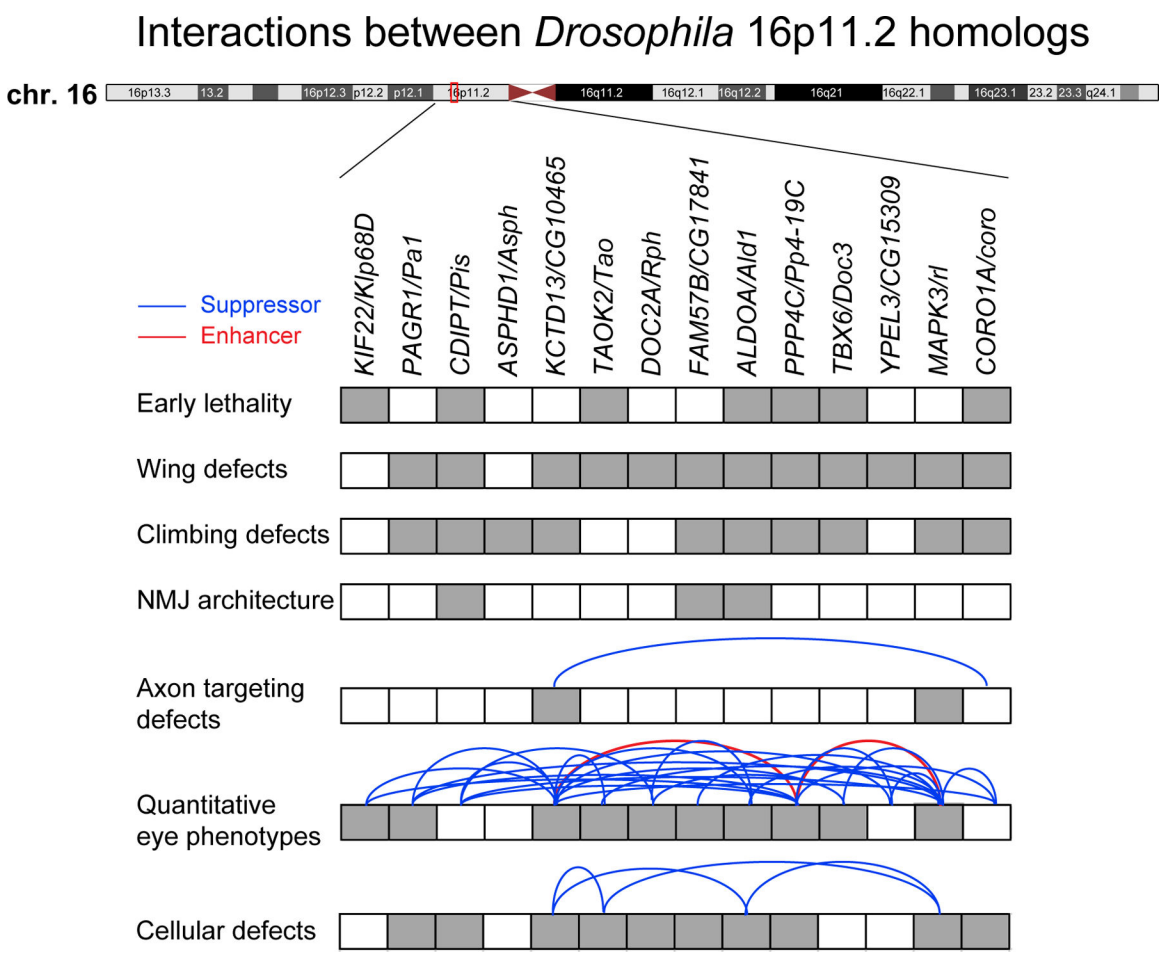

Figure 1. *Drosophila* homologs of 16p11.2 genes exhibit complex genetic interactions.

Summary of 14 fly homologs of 16p11.2 genes for developmental, neuronal, and cellular phenotypes. Shaded gray boxes indicated presence of a phenotype, and the lines (red and blue) connecting the genes above the boxes indicate interaction patterns between homologs of 16p11.2 genes.

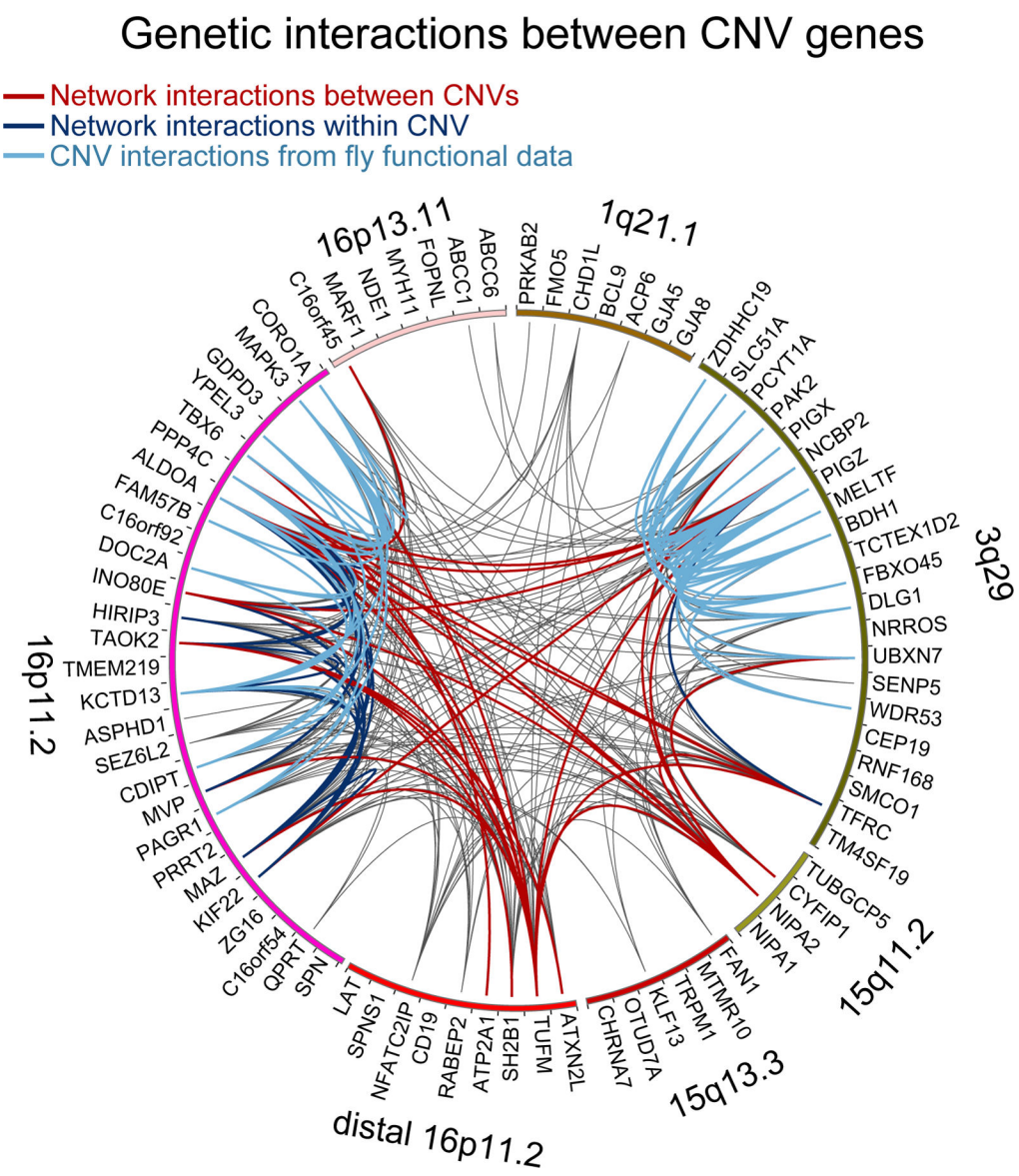

Figure 2. Genetic interactions between CNV genes.

Circos plot shows predicted interactions of genes within seven CNV regions in a human brain-specific interaction network, along with functionally-validated gene interactions from *Drosophila* experiments. Thin black lines represent all connections between CNV genes in the network (n=255). Highlighted interactions are the top 50 strongest interactions in the network, including red lines that represent interactions between different CNVs and dark blue lines that represent interactions within the same CNV. Light blue lines represent 50 interactions that were separately identified using *Drosophila* experiments.

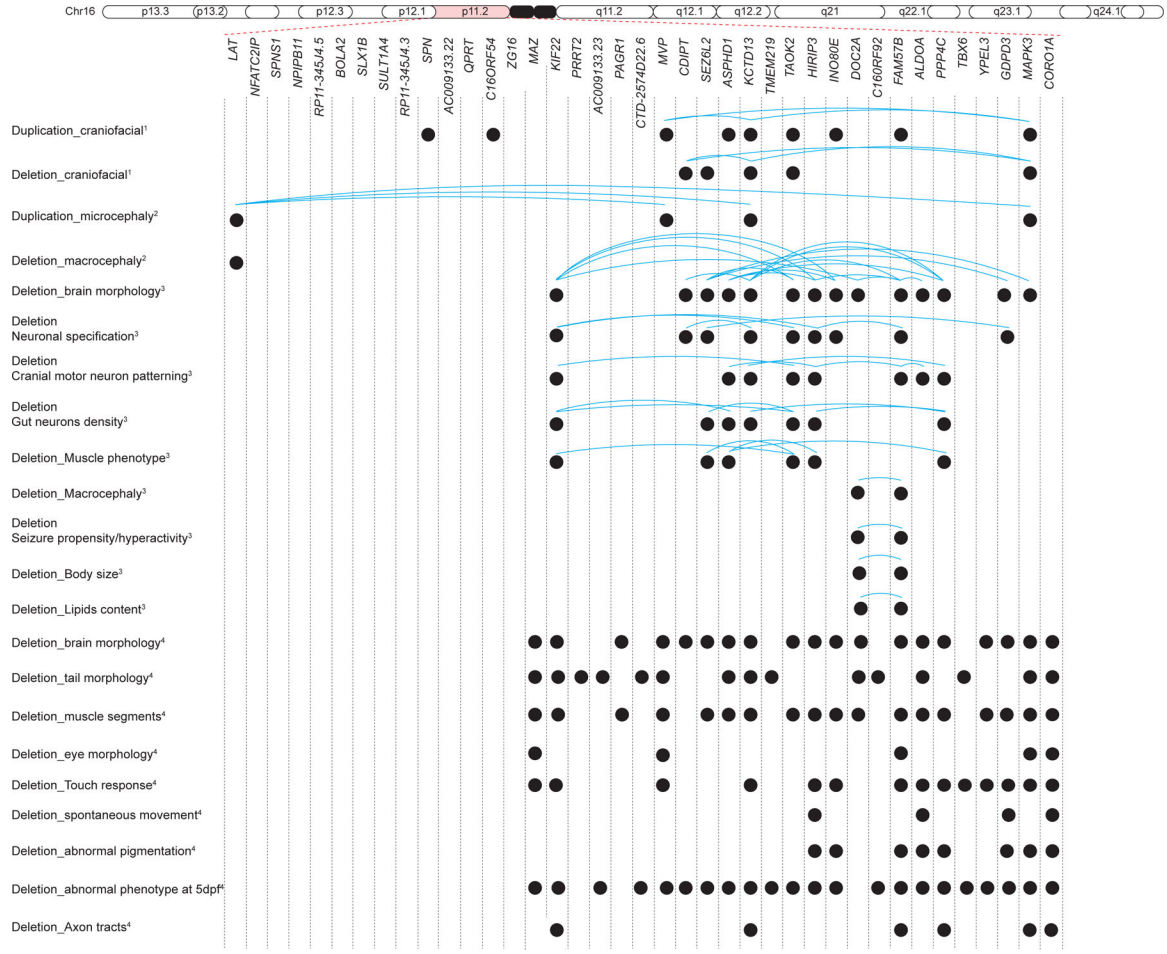

Figure 3. *Zebrafish* homologs of 16p11.2 genes exhibit single genetic drivers and complex genetic interactions.

Summary of 39 16p11.2 genes for neuroanatomical, craniofacial, gross morphological, and tissue-specific phenotypes in zebrafish. Black dots indicated presence of a phenotype, and lines (blue) connecting the genes above the dots indicate interactions between 16p11.2 genes.