Drosophila models of pathogenic copy-number variant genes show global and non-neuronal defects during development

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ABSTRACT

While rare pathogenic CNVs are associated with both neuronal and non-neuronal phenotypes, functional studies evaluating these regions have focused on the molecular basis of neuronal defects. We report a systematic functional analysis of non-neuronal phenotypes for 59 homologs of genes within ten CNVs and 20 neurodevelopmental genes in *Drosophila*. Using wing-specific knockdown of 136 RNA interference lines, we identify phenotypes in 72/79 homologs including six lines with lethality and 21 lines with severe phenotypes. We find no correlation between severity of these phenotypes and neuronal defects due to eye-specific knockdown. We observe disruptions in cell proliferation and apoptosis for 23/27 homologs, and altered Wnt, Hedgehog and Notch signaling for 9/14 homologs, including *AATF/Aatf*, *PPP4C/Pp4-19C*, and *KIF11/Klp61F,* validated with differences in human tissue-specific expression and network connectivity. Our findings suggest that multiple genes within each CNV differentially affect both global and tissue-specific developmental processes, contributing to non-neuronal phenotypes of CNV disorders.

INTRODUCTION

Rare copy-number variants (CNVs), or deletions and duplications in the genome, are associated with neurodevelopmental disorders such as autism, intellectual disability (ID), and schizophrenia[1,2]. While dosage alteration of CNV regions contribute predominantly to defects in nervous system development, several CNV disorders also lead to early developmental features involving other organ systems[3,4], including cardiac defects[5,6], kidney malformations[7], craniofacial features[3], and skeletal abnormalities[8]. In fact, an overall survey of ten rare disease-associated CNVs among individuals within the DECIPHER database[9] showed a wide range of non-neuronal phenotypes across multiple organ systems for each CNV disorder (**Fig. 1**). For example, the 1q21.1 deletion causes variable expression of multiple neuronal and non-neuronal phenotypes, including developmental delay, autism, and schizophrenia as well as craniofacial features, cataracts, cardiac defects, and skeletal abnormalities[10–12]. Additionally, while the 7q11.23 deletion associated with Williams-Beuren syndrome (WBS) causes neuropsychiatric and behavioral features, other non-neuronal phenotypes, including supravalvular aortic stenosis, auditory defects, hypertension, diabetes mellitus, and musculoskeletal and connective tissue anomalies, are also observed among the deletion carriers[13]. In fact, individual genes within the WBS region are associated with specific features of the deletion, such as *ELN* and supravalvular aortic stenosis[14], *STX1A* and impaired glucose tolerance[15], *LIMK1* and impaired visuospatial abilities[16], and *GTF2IRD1* and craniofacial abnormalities[17]. Furthermore, *TBX1* was identified to cause the pharyngeal arch cardiac defects associated with the 22q11.2 deletion (DiGeorge syndrome)[18], while *HNF1B* within the 17q12 deletion region was identified as the causative gene for kidney defects associated with the deletion[19,20]. However, candidate genes for a majority of non-neuronal phenotypes have not been identified for several rare CNV disorders, in particular

for CNVs associated with variably-expressive phenotypes such as the 1q21.1 deletion and the 16p11.2 deletion[21,22]. Additionally, affected individuals who carry disruptive mutations in neurodevelopmental genes from recent sequencing studies have also been documented to manifest non-neuronal phenotypes. For example, individuals with loss-of-function mutations in the autism-associated gene *CHD8* present with gastrointestinal problems, tall stature, and craniofacial features in addition to neuropsychiatric features[23], while mutations in the microcephaly-associated gene *KIF11* also lead to congenital lymphedema and retinopathy[24].

Despite the importance of identifying genes within CNVs that contribute towards non-neuronal phenotypes, functional studies of CNV genes have primarily focused on detailed assessments of neuronal phenotypes in model systems. For example, mouse models generated for the 16p11.2 deletion exhibited post-natal lethality, reduced brain size and neural progenitor cell count, motor and habituation defects, synaptic defects, and behavioral defects[25–27]. Similarly, mouse models for the 3q29 deletion showed decreased weight and brain size, increased locomotor activity and startle response, and decreased spatial learning and memory[28,29]. However, fewer studies have focused on detailed evaluation of non-neuronal phenotypes in functional models of CNV disorders. For example, Arbogast and colleagues evaluated obesity and metabolic changes in 16p11.2 deletion mice, which showed reduced weight and impaired adipogenesis[30]. While Haller and colleagues showed that mice with knockdown of *MAZ,* a gene within the 16p11.2 deletion region, contribute to the genitourinary defects observed in individuals with the deletion[31], mouse studies on other homologs of 16p11.2 genes, including *TAOK2*, *KCTD13*, and *MAPK3,* have only focused on assessing neuronal defects[32–36]. Furthermore, Dickinson and colleagues reported a high-throughput analysis of essential genes in mice and identified both neuronal and non-neuronal phenotypes for individual gene knockouts,

including more than 400 genes that lead to lethality[37]. While these efforts aided in implicating novel genes with human disease, our understanding of how genes associated with neurodevelopmental disorders contribute towards non-neuronal phenotypes is still limited. Therefore, a large-scale analysis of non-neuronal phenotypes is necessary to identify specific candidate genes within CNV regions and associated biological mechanisms that contribute towards these phenotypes.

Drosophila melanogaster is an excellent model system to evaluate homologs of neurodevelopmental genes, as many developmental processes and signaling pathways are conserved between humans and flies[38]. In fact, over 75% of human disease genes have homologs in *Drosophila*, including many genes involved in cellular signaling processes[39,40]. We recently examined the contributions of individual *Drosophila* homologs of 28 genes within the 16p11.2 and 3q29 deletion regions towards specific neurodevelopmental phenotypes, including rough eye phenotypes and defects in climbing ability, axon targeting, neuromuscular junction, and dendritic arborization[41,42]. While these findings implicated multiple genes within each CNV region towards neuronal phenotypes, the conserved role of these genes towards non-neuronal phenotypes is not well understood. The *Drosophila* wing is an effective model system to evaluate such developmental phenotypes, as key components of conserved signaling pathways, such as Notch, epidermal growth factor receptor (EGFR), Hegdehog, and Wnt pathways, were identified using fly wing models[43–49]. For example, Wu and colleagues showed that overexpression of the *Drosophila* homolog for *UBE3A*, associated with Angelman syndrome, leads to abnormal wing and eye morphology defects[50]. Furthermore, *Drosophila* mutant screens for developmental phenotypes, including wing defects, were used to identify conserved genes for several human genetic diseases, including Charcot-Marie-Tooth disease and syndromic

109 microcephaly[51]. Kochinke and colleagues also recently performed a large-scale screening of ID-
110 associated genes, and found an enrichment of wing trichome density and missing vein
111 phenotypes in ID genes compared to control gene sets[52]. Hence, the fly wing provides a model
112 system that is ideal for evaluating the contributions of individual homologs of CNV genes
113 towards cellular and developmental phenotypes.

114 In this study, we tested the non-neuronal phenotypes of 79 fly homologs of human genes
115 within ten pathogenic CNV regions and genes associated with neurodevelopmental disorders.
116 We observed a wide range of robust qualitative and quantitative adult wing phenotypes among
117 the 136 RNA interference (RNAi) lines tested in our study, including size defects, ectopic and
118 missing veins, severe wrinkling, and lethality. Further analysis of cellular phenotypes revealed
119 disruptions in conserved developmental processes in the larval imaginal wing disc, including
120 altered levels of cell proliferation and apoptosis as well as altered expression patterns in the Wnt,
121 Hedgehog, and Notch signaling pathways. However, we found no correlation in the severity of
122 phenotypes observed with wing and eye-specific knockdown. Our findings were further
123 supported by differences in expression patterns and network connectivity of human CNV genes
124 across different tissues. Our analysis emphasizes the importance of multiple genes within each
125 CNV region towards both global and tissue-specific developmental processes, potentially
126 accounting for the non-neuronal phenotypes associated with pathogenic CNVs.
127

RESULTS

Wing-specific knockdown of fly homologs of CNV genes show non-neuronal phenotypes

Using an RNAi based analysis driven by the *bx*^{MS1096}*-GAL4* wing-specific driver, we tested a total of 136 RNAi lines for 59 homologs of genes within pathogenic CNV regions (chromosomal locations 1q21.1, 3q29, 7q11.23, 15q11.2, 15q13.3, 16p11.2, distal 16p11.2, 16p12.1, 16p13.11, and 17q12) and 20 homologs of genes associated with neurodevelopmental disorders (**Supp. Data 1**). Fly homologs of these genes were identified using the DIOPT orthology prediction tool[53]. We list both the human gene name and the fly gene name for each tested gene as *HUMAN GENE/Fly gene* (i.e. *KCTD13/CG10465*) as well as the human CNV region for context at first instance. We scored 20-25 adult wings for five distinct wing phenotypes in each non-lethal RNAi line, including wrinkled wing, discoloration, ectopic veins, missing veins, and bristle planar polarity phenotypes (**Fig. 2A; Supp. Data 2**). We first categorized each wing phenotype based on their severity and performed k-means clustering analysis to categorize each RNAi line by their overall phenotype severity (**Fig. 2B-C**). We observed four clusters of RNAi lines: 75 lines with no observable qualitative phenotypes (55.2%), 24 lines with mild phenotypes (17.7%), 10 lines with moderate phenotypes (7.4%), 21 lines with severe phenotypes (15.4%), and 6 lines with lethal phenotypes (4.4%), including *ACACA/ACC* within 17q12, *DLG1/dlg1* within 3q29, and *STX1A/Syx1A* within 7q11.23 (**Fig. 2B-D; Supp. Data 2**). We observed severe wrinkled wing phenotypes for 13/79 fly homologs, including *PPP4C/Pp4-19C* within 16p11.2, *ATXN2L/Atx2* within distal 16p11.2, *AATF/Aatf* within 17q12, and *MFI2/Tsf2* within 3q29 (**Fig. 3A-B, Supp. Data 3**). Interestingly, seven out of ten CNV regions contained at least one homolog that showed lethality or severe wing phenotypes, and five CNV regions (3q29, 16p11.2, distal 16p11.2, 16p12.1, and 17q12) had multiple homologs showing lethality or severe wing

phenotypes (**Fig. 3A, Supp. Data 3**). For example, RNAi lines for both *UQCRC2/UQCR-C2* and *POLR3E/Sin* within 16p12.1 showed lethality. Within the 3q29 region, *NCBP2/Cbp20* and *MFI2/Tsf2* showed severe phenotypes while *DLG1/dlg1* showed lethality. In contrast, 12/20 known neurodevelopmental genes showed no observable wing phenotypes, suggesting that these genes could be responsible for neuronal-specific phenotypes (**Fig. 3B, Supp. Data 3**). We note that 18/79 fly homologs showed discordant phenotypes between two or more RNAi lines for the same gene, which could be due to differences in expression of the RNAi construct among these lines (**Supp. Data 3**).

Certain qualitative phenotypes exhibited higher frequency in males compared to females. For example, discoloration (87 lines in males compared to 56 lines in females; $p=1.315\times10^{-4}$, two-tailed Fisher's exact test) and missing vein phenotypes (92 lines in males compared to 29 lines in females; $p=2.848\times10^{-16}$, two-tailed Fisher's exact test) at any degree of severity were more commonly observed in males than females (**Supp. Data 2**). In particular, 25/92 lines in males (compared to 1/29 in females) showed a total loss of the anterior crossvein (ACV) (**Supp. Data 2**). We further identified 17 RNAi lines that were lethal in males with wing-specific knockdown of fly homologs. While higher frequencies of wing phenotypes in males could be due to a sex-specific bias of developmental phenotypes, the increased severity we observed in males is most likely due to a stronger RNAi knockdown caused by an X-linked dosage compensation, as the *bx*^{MS1096}*-GAL4* driver is inserted on the fly X chromosome[54,55].

Next, we measured the total adult wing area and the lengths of six veins (longitudinal L2, L3, L4, L5, ACV, and posterior crossvein or PCV) in the adult wing for each of the tested RNAi lines that did not show lethality (or severe wrinkled phenotypes for vein length measurements) (**Fig. 4A**). Overall, we identified significant wing measurement changes for 89 RNAi lines

compared to controls, which included lines that did not have an observable qualitative wing phenotype (**Fig. 2D**). A summary of L3 vein lengths is presented in **Fig. 4B**, and the measurements for the remaining five veins are presented in **Supp. Figure 1** and **Supp. Data. 2**. We found that 33/61 of the homologs (54%) showed significant changes in L3 vein length, including 20 homologs with longer vein lengths and 13 homologs with shorter vein lengths (**Supp. Data 3**). Additionally, 41/74 of the fly homologs (55%) showed changes in wing area (**Supp. Data 3**), including 36 homologs which showed smaller wing areas and five homologs showed larger wing areas compared to controls (**Supp. Data 3**). For example, both homologs of genes within 1q21.1 region, *BCL9/lgs* and *FMO5/Fmo-2*, showed decreased wing area and vein length, potentially mirroring the reduced body length phenotype observed in mouse models of the deletion[56] (**Fig. 4B-C**). In addition, *PAK2/Pak* within 3q29*, TBX1/org-1* within 22q11.2, autism-associated *CHD8/kis*, and microcephaly-associated *ASPM/asp* also showed smaller wing areas and vein lengths (**Fig. 4B-C**). In contrast, *TRPM1/Trpm* within 15q13.3 and the cell proliferation gene *PTEN/Pten*[57] both showed larger wing areas and vein lengths (**Fig. 4B-C**). Furthermore, we identified eight homologs that showed no qualitative wing phenotypes but had significant changes in wing areas and vein lengths (**Supp. Data 3**), including *CCDC101/Sgf29* in distal 16p11.2*, FMO5/Fmo-2, TRPM1/Trpm, DHRS11/CG9150* in 17q12, and *NSUN5/Nsun5* in 7q11.23 (**Fig. 4B-C; Supp. Data 3**). These results indicate that homologs of certain CNV genes may influence variations in size without causing adverse wing phenotypes, and may be specifically implicated towards cellular growth mechanisms.

Homologs of CNV genes show global and tissue-specific effects during development

We previously showed that many of the same fly homologs of CNV genes that showed wing defects in the current study also contributed towards neuronal phenotypes in the fly eye[41,42], suggesting a role for these genes in global development. We therefore performed ubiquitous and eye-specific knockdown of fly homologs to assess tissue-specific effects in comparison to the wing phenotypes. First, we used the *da-GAL4* driver at 25°C to drive ubiquitous knockdown of RNAi lines for 31 homologs of CNV genes, including 19 that were previously published[41,42], and observed complete or partial lethality at larval and pupal stages with knockdown of 10/31 homologs (32.3%) (**Fig. 5A**). Lethal phenotypes have also been documented for 43/130 knockout mouse models of individual CNV genes as well as for the entire deletion (**Supp. Data 4**). For example, mouse models heterozygous for the 16p11.2 deletion showed partial neonatal lethality, while knockout mouse models of four individual genes within the 16p11.2 region, including *Ppp4C*$^{-/-}$ and *Kif22*$^{-/-}$, showed embryonic lethality[25,58,59]. In our study, the *DLG1/dlg1* line that showed lethality with wing-specific knockdown also exhibited larval lethality with ubiquitous knockdown, indicating its role in global development (**Fig. 5A**). In addition, six homologs that showed severe wing phenotypes also showed larval or pupal lethality with ubiquitous knockdown, including *ALDOA/Ald* and *PPP4C/Pp4-19C* within 16p11.2 and *ATXN2L/Atx2* and *TUFM/mEFTu1* within distal 16p11.2 (**Fig. 5A**). The remaining homologs that showed lethality with ubiquitous knockdown showed at least a mild qualitative or quantitative wing phenotype.

We next compared the phenotypes observed with wing-specific knockdown of fly homologs to their corresponding eye-specific knockdowns to evaluate neuronal versus non-neuronal effects. To quantitatively assess the phenotypic severity of cellular defects with eye-

specific knockdown of fly homologs, we developed a tool called *Flynotyper*[60] that determines the degree of disorganization among the ommatidia in the adult eye. We analyzed phenotypic scores obtained from *Flynotyper* for 66 RNAi lines of 45 fly homologs, including from previously-published datasets[41,42,60]. We found that 37/45 homologs (82.2%) exhibited both eye and wing-specific defects (**Fig. 5B, Supp. Fig. 2, Supp. Data 5**). Two homologs with significant eye phenotypes did not show any wing phenotypes, including *SPNS1/spin* within distal 16p11.2 and microcephaly-associated *SLC25A19/Tpc1*[61], while five homologs only showed wing-specific phenotypes, including *CDIPT/Pis* and *YPEL3/CG15309* within 16p11.2, *FBXO45/Fsn* and *OSTalpha/CG6836* within 3q29, and *UQCRC2/UQCR-C2* (**Fig. 5B, Supp. Fig. 2**). In particular, *UQCRC2/UQCR-C2* showed lethality with wing-specific knockdown, suggesting potential tissue-specific effects of this gene in non-neuronal cells (**Fig. 5B**). While most homologs contributed towards both eye and wing-specific phenotypes, we observed a wide range of severity in eye phenotypes that did not correlate with the severity of quantitative or qualitative wing phenotypes (**Fig. 5C**). For example, *TUFM/mEFTu1* showed a severe wing phenotype but only a mild increase in eye phenotypic score, while *SH2B1/Lnk,* also within the distal 16p11.2 region, showed severe rough eye phenotypes but only a mild increase in wing size (**Fig. 5D**). Similarly, *BCL9/lgs* also showed opposing tissue-specific effects with mild qualitative wing phenotype and severe eye phenotype, suggesting that the role of these homologs towards development differs across tissue types.

CNV genes show variable expression across different tissues in flies and humans

To assess how expression levels of CNV genes vary across different tissues, we first examined the expression patterns of fly homologs in larval and adult tissues using the FlyAtlas Anatomical

Microarray dataset[62]. We found that 76/77 homologs with available data were expressed in at least one larval and adult tissue (**Supp. Fig. 3, Supp. Data 6**). In general, we did not observe a correlation between wing phenotype severity and expression patterns of homologs in larval or adult tissues (**Fig. 6A**). For example, 58/77 homologs (75.3%) showed ubiquitous larval expression, including both fly homologs that showed no qualitative wing phenotypes, such as *KCTD13/CG10465* within 16p11.2 and *FBXO45/Fsn*, and those with severe wing phenotypes, such as *PPP4C/Pp4-19C* and *NCBP2/Cbp20* (**Fig. 6A, Supp. Fig. 3**). Furthermore, 30/39 homologs (76.9%) that showed eye phenotypes also had ubiquitous larval expression, providing further support to the observation that genes causing neuronal phenotypes may also contribute to developmental phenotypes in other tissues (**Supp. Data 5**). Of note, 9/77 homologs (11.7%) did not have any expression in the larval central nervous system, including *FMO5/Fmo-2*, *BDH1/CG8888* within 3q29, and *TBX6/Doc2* within 16p11.2 (**Fig. 6A, Supp. Fig. 3**). However, we observed wing phenotypes for 8/9 of these homologs, suggesting that they may contribute to tissue-specific phenotypes outside of the nervous system. Except for the epilepsy-associated *SCN1A/para*[63], which was exclusively expressed in both the larval central nervous system (CNS) and adult brain tissues, other tested neurodevelopmental genes were also expressed in non-neuronal tissues (**Fig. 6A**).

We further used the GTEx Consortium dataset[64] to examine tissue-specific expression of 150 human CNV and known neurodevelopmental genes across six tissues including brain, heart, kidney, lung, liver, and muscle. We found 121 genes that were expressed in at least one adult tissue, including 49 genes (32.7%) that showed ubiquitous expression across all six tissues (**Supp. Data 6**). Of the 112 genes expressed in non-neuronal tissues, 34 did not have any neuronal expression, including *TBX1*, *FMO5* and *GJA5* within 1q21.1, and *ATP2A1* within distal

16p11.2 (**Fig. 6B**, **Supp. Data 6**). *FMO5* and *TBX1* also showed non-neuronal expression in *Drosophila* tissues, suggesting that their tissue-specific expression is highly conserved (**Fig. 6A**). Other genes showing ubiquitous expression also had preferentially high expression for specific non-neuronal tissues, including *ALDOA* and *UQCRC2* for muscle and heart for (**Fig. 6B**). In contrast, we found nine genes that were expressed only in the adult brain, including *FAM57B* and *DOC2A* within 16p11.2, as well as *SCN1A*, which showed similar CNS-only expression in *Drosophila* tissues (**Fig. 6B**, **Supp. Data 6**).

Knockdown of fly homologs of CNV genes lead to disruption of basic cellular processes

The disruption of basic cellular processes in neuronal cells, such as cell proliferation and apoptosis, have been implicated in neurodevelopmental disorders[65–67]. We previously identified defects in cell proliferation among photoreceptors neurons in larval eye discs with knockdown of 16p11.2 homologs, as well as increased apoptosis with knockdown of a subset of 3q29 homologs[41,42]. Here, we explored how these basic cellular processes are altered in non-neuronal cells, specifically in the developing wing disc. We targeted 27 fly homologs that showed a range of adult wing phenotypes for changes in cell proliferation and apoptosis, using anti-phospho-Histone H3 Ser10 (pH3) and anti-*Drosophila* caspase-1 (dcp1), respectively, in the third instar larval wing discs. We identified 23/27 homologs that showed significant increases in apoptotic cells compared to controls, including seven homologs, such as *PPP4C/Pp4-19C*, *ATXN2L/Atx2*, and *AATF/Aatf* , which showed dcp1 staining across the entire larval wing pouch (**Fig. 7A-B, Supp. Figs. 4-5, Supp. Data 7**). In addition, 16/27 genes showed decreased levels of proliferation, including eight homologs which also showed apoptosis defects, such as *CYFIP1/Sra-1* within 15q11.2, *SH2B1/Lnk*, and the microcephaly gene *KIF11/Klp61F* (**Fig. 7A**

289 **and 7C, Supp. Figs. 4-5, Supp. Data 7**). All six of the tested homologs with severe adult wing
290 phenotypes showed both increased apoptosis and decreased proliferation (**Supp. Data 7**).
291 Similarly, 3/4 homologs of genes showing lethality with wing-specific knockdown also showed
292 defects in apoptosis or proliferation, with the exception of *ACACA/ACC* (**Supp. Figure 4**, **Supp.**
293 **Data 7**). As *bx*^{MS1096}-*GAL4* is located on the X-chromosome, we expected to see more severe
294 defects in males compared with females with knockdown of homologs due to the X-linked
295 dosage compensation[54,55]. However, knockdown of 3/11 tested homologs with sex-specific
296 differences in adult wing phenotypes, including *BCL9/lgs*, *CYFIP1/Sra-1*, and
297 *DNAJC30/CG11035* within 7q11.23, showed significantly decreased levels of cell proliferation
298 in females but no change for males compared to their respective controls, suggesting a sex-
299 specific effect of these genes for cell proliferation (**Supp. Fig. 5, Supp. Data 7**). Overall, our
300 results suggest that cell proliferation and apoptosis play an important role towards development
301 in both neuronal and non-neuronal tissues.
302
303 **Homologs of candidate CNV genes disrupt conserved signaling pathways**
304 Several conserved signaling pathways that are active in a spatial and temporal manner in the
305 larval wing disc, such as Wnt, Hedgehog, BMP, and Notch signaling, regulate the anterior-
306 posterior (A/P) and dorsal-ventral (D/V) boundaries to determine accurate morphology and vein
307 patterning in the adult wing[48,49,68–70]. For example, Wnt and Notch signaling pathways both act
308 along the D/V boundary to determine cell fate[71,72], while Hedgehog signaling is dependent upon
309 expression of both engrailed in the posterior compartment and patched along the A/P border[73,74].
310 Furthermore, O'Roak and colleagues showed that genes identified from *de novo* mutations in
311 patients with autism are linked to β-catenin/Wnt pathway[75]. In addition, familial loss-of-function

312 mutations in the human hedgehog signaling pathway gene *PTCH1* are implicated in basal cell
313 nevus syndrome, leading to basal cell carcinoma[76,77].

314 Based on adult wing phenotypes and disruptions to cellular processes, we next tested
315 whether knockdown of 14 fly homologs disrupt conserved signaling pathways in the third instar
316 larval wing disc (**Supp. Data 7**). In particular, we evaluated the role of Wnt, Hedgehog, and
317 Notch signaling pathways by testing the expression patterns of four key proteins within these
318 pathways, including wingless (Wnt), patched (Hedgehog), engrailed (Hedgehog), and delta
319 (Notch). We found that 9/14 homologs, including 8/10 homologs showing severe wing
320 phenotypes or lethality, exhibited disruptions in at least one signaling pathway. For example, five
321 homologs with severe or lethal phenotypes showed disruptions of all four signaling pathways,
322 including *AATF/Aatf*, *NCBP2/Cbp20*, *POLR3E/Sin*, *PPP4C/Pp4-19C*, and *KIF11/Klp61F* (**Fig.**
323 **8, Supp. Data 7**). Our observations are in concordance with previous findings by Swarup and
324 colleagues, who showed that *PPP4C/Pp4-19C* is a candidate regulator of Wnt and Notch
325 signaling pathways in *Drosophila* larval wing discs[78]. Furthermore, two genes from the 3q29
326 region, *DLG1/dlg1* and *MFI2/Tsf2*, showed altered expression patterns for delta and patched but
327 not for engrailed, indicating that they selectively interact with the Hedgehog as well as Notch
328 signaling pathway (**Supp. Fig. 6**). In fact, Six and colleagues showed that Dlg1 directly binds to
329 the PDZ-binding domain of Delta1[79]. In contrast, *ACACA/ACC* and *UQCRC2/UQCR-C2* showed
330 no changes in expression patterns for any of the four signaling proteins tested, suggesting that the
331 observed lethality could be due to other cellular mechanisms (**Supp. Fig. 6**). We conclude that a
332 subset of homologs disrupt the expression of key proteins in signaling pathways, potentially
333 accounting for the developmental phenotypes observed in the adult wings.
334

335 **Connectivity patterns of candidate genes vary across human tissue-specific networks**

336 We examined patterns of connectivity for the nine candidate genes which showed disruptions of
337 signaling pathways within the context of human brain, heart, and kidney-specific gene
338 interaction networks[80]. These tissue-specific networks were constructed using Bayesian
339 classifier-generated probabilities for pairwise genetic interactions based on co-expression data[80].
340 We calculated the lengths of the shortest paths between each candidate gene and 267 Wnt,
341 Notch, and Hedgehog pathway genes in each network as a proxy for connectivity (**Supp. Data**
342 **8**). In all three networks, each of the candidate genes were connected to a majority of the tested
343 signaling pathway genes, suggesting that our results have translational relevance towards human
344 developmental pathways (**Fig. 9A, Supp. Fig. 7**). Interestingly, we observed a higher
345 connectivity (i.e. shorter path distances) between candidate genes and Wnt and Hedgehog
346 pathway genes in the brain-specific network compared to the heart and kidney-specific networks
347 (**Fig. 9B**). We further identified enrichments for genes involved in specific biological processes
348 among the connector genes that were located in the shortest paths within neuronal and non-
349 neuronal tissue-specific networks (**Fig. 9C, Supp. Data 8**). For example, axon-dendrite
350 transport, dopaminergic signaling, and signal transduction functions were enriched among
351 connector genes only for the brain-specific network, while organelle organization and protein
352 ubiquitination were enriched among connector genes only for kidney and heart networks (**Fig.**
353 **9C**). However, several core biological processes, such as cell cycle, protein metabolism,
354 transcriptional regulation, and RNA processing/splicing, were enriched among connector genes
355 within all three tissue-specific networks (**Fig. 9C**). Our analysis highlights that human CNV
356 genes potentially interact with developmental signaling pathways in an ubiquitous manner, but
357 may affect different biological processes in neuronal and non-neuronal tissues.

DISCUSSION

We used the *Drosophila* wing as a model to identify key CNV genes involved in non-neuronal phenotypes associated with CNV disorders. We tested fly homologs of 79 genes and identified multiple homologs within each CNV region that exhibited strong phenotypes indicative of developmental disruptions. Several themes have emerged from our study highlighting the importance of fly homologs of CNV genes towards both global and tissue-specific phenotypes associated with human CNV disorders.

First, we found that homologs of CNV genes contribute towards developmental phenotypes through ubiquitous roles in neuronal and non-neuronal tissues. Although we did not study models for the entire CNV, nearly all individual fly homologs of CNV genes contribute to wing-specific developmental phenotypes. It is likely that these genes may also contribute to additional phenotypes in other tissues that we did not assess. In fact, a subset of these genes also showed early lethality with ubiquitous knockdown in addition to severe or lethal wing-specific phenotypes. However, we found no correlation between the severity of the eye and wing phenotypes, suggesting tissue-specific effects of these homologs towards developmental phenotypes. In contrast, fly homologs of known neurodevelopmental genes generally showed milder wing phenotypes compared with eye phenotypes, indicating a more neuronal role for these genes. While our study only examined a subset of CNV genes with *Drosophila* homologs, phenotypic data from knockout mouse models also support a global developmental role for individual CNV genes. In fact, 44/130 knockout models of CNV genes within the Mouse Genome Informatics (MGI) database[81] exhibited non-neuronal phenotypes, including 20 homologs of CNV genes that showed both neuronal and non-neuronal phenotypes (**Supp. Data 4**). For example, knockout mouse models of *Dlg1*$^{-/-}$ show defects in dendritic growth and

branching in the developing nervous system, in addition to craniofacial features and multiple kidney and urinary tract defects[82–85]. Furthermore, Chapman and colleagues showed that knockout of *Tbx6*$^{-/-}$ caused defects in mesodermal and neuronal differentiation early in development, leading to abnormal vascular, tail bud, and neural tube morphology[86]. These observations further support our findings that most fly homologs of CNV genes have a global role in development that could account for the observed non-neuronal phenotypes.

Second, based on tissue-specific phenotypes, we identified fly homologs of CNV genes that are key regulators of conserved cellular processes important for development. For example, 9/10 homologs with severe or lethal adult wing phenotypes also exhibited defects in cell proliferation and apoptosis during development. In fact, we found concordance between cellular processes affected by wing and eye-specific knockdown of homologs of genes within 16p11.2 and 3q29 regions, including decreased proliferation for *MAPK3/rl* and increased apoptosis for *NCBP2/Cbp20* and *DLG1/dlg1*[41,42]. While eye-specific knockdown of *BDH1/CG8888* showed decreased cell proliferation in larval eye discs[42], we found increased cell proliferation with wing-specific knockdown, suggesting a tissue-specific effect for this gene. Notably, at least one fly homolog per CNV region showed defects in cell proliferation or apoptosis, suggesting that these conserved cellular processes may be relevant to CNV pathogenicity. For example, *ATXN2L/Atx2*, *SH2B1/Lnk*, and *CCDC101/Sgf29* each showed decreased proliferation and increased apoptosis, suggesting an underlying common conserved cellular mechanism for the distal 16p11.2 deletion. Furthermore, a subset of these genes also disrupted multiple signaling pathways, indicating a potential role for these genes as key regulators of developmental processes. We specifically identified five genes whose knockdown caused disruptions of Wnt, Notch, and hedgehog signaling pathways. Each of these genes have important roles in cell cycle regulation, apoptosis,

transcription, or RNA processing, based on Gene Ontology annotations[87,88]. In fact, we found that the RNA transport protein *NCBP2/Cbp20*[89], which we recently identified as a key modifier gene for the 3q29 deletion[42], interfaced with all three signaling pathways. Furthermore, *AATF* disrupts apoptosis and promotes cell cycle progression through displacement of HDAC1[90–92], while *PPP4C* promotes spindle organization at the centromeres during mitosis[93]. While we only evaluated the role of these genes towards development in a single fly tissue, our additional analysis of human gene interaction networks showed strong connectivity between the CNV genes and signaling pathways in multiple neuronal and non-neuronal human tissues. In fact, cell cycle genes were enriched among the connector genes in all three tissue-specific networks, further emphasizing the role of cell cycle processes towards developmental phenotypes. Notably, we also observed certain biological processes enriched among connector genes that were specific to neuronal or non-neuronal tissues, indicating that haploinsufficiency of genes within CNV regions may disrupt different biological processes in a tissue-specific manner.

Overall, we show that fly homologs of most CNV genes contribute towards global developmental phenotypes, although exactly how they contribute toward such phenotypes varies between neuronal and non-neuronal tissues. Previous functional studies for CNV disorders have focused primarily on identifying candidate genes for the observed neuronal phenotypes. In this study, we identified several homologs of CNV genes that are responsible for non-neuronal phenotypes, as well as novel associations between these genes and conserved biological processes and pathways. We therefore propose that multiple genes within each CNV disrupt global and tissue-specific processes during development and contribute to the wide range of non-neuronal phenotypes associated with CNV disorders (**Fig. 10**). This multigenic model for non-neuronal phenotypes in CNV disorders is in line with our previous model for neuronal

phenotypes of these disorders, as opposed to models where individual causative genes are responsible for specific phenotypes[41,42,94]. Our study further exemplifies the utility of evaluating non-neuronal phenotypes in addition to neuronal phenotypes in models of individual genes and CNV regions associated with developmental disorders, including future studies in mammalian or cellular model systems. Further studies exploring how CNV genes interact with each other and with other developmental pathways could more fully explain the conserved mechanisms underlying global developmental defects and identify potential therapeutic targets for these disorders.

METHODS

Fly stocks and genetics

We tested 59 *Drosophila* homologs for 130 human genes that span across 10 pathogenic CNV regions associated with neurodevelopmental disorders (1q21.1, 3q29, 7q11.23, 15q11.2, 15q13.3, 16p11.2, distal 16p11.2, 16p12.1, 16p13.11, and 17q12)[22] (**Supp. Data 1**). In addition, we evaluated fly homologs of 20 human genes known to be in involved in neurodevelopmental disorders[60,95] (**Supp. Data 1**). These include genes involved in beta-catenin signaling pathway (5 genes), core genes implicated in neurodevelopmental disorders (8 genes), and genes associated with microcephaly (7 genes)[96]. We used the DRSC Integrative Ortholog Prediction Tool (DIOPT, v.7.1) to identify the fly homologs for each human gene[53] (**Supp. Data 1**).

To knockdown individual genes in specific tissues, we used RNA interference (RNAi) and the *UAS-GAL4* system (**Fig. 2A**), a well-established tool that allows for tissue-specific expression of a gene of interest[97]. RNAi lines were obtained from Vienna *Drosophila* Resource Center (VDRC) that include both GD and KK lines. We tested a total of 136 lines in our final data analysis (**Supp. Data 9**), after eliminating KK lines with additional insertion that drives the overexpression of the Tiptop (*tio*) transcription factor[98,99]. A complete list of stock numbers and full genotypes for all RNAi lines used in this study is presented in **Supp. Data 9**. We used the *bx^{MS1096}-GAL4/FM7c;;UAS-Dicer2/TM6B* driver for wing-specific knockdown and *w^{1118};GMR-GAL4*;*UAS-Dicer2* driver (Claire Thomas, Penn State University) for eye-specific knockdown of RNAi lines. Ubiquitous knockdown experiments were performed using the *w;da-GAL4;+* driver (Scott Selleck, Penn State University). For all experiments, we used appropriate GD (*w^{1118}*, VDRC# 60000) or KK (*y,w^{1118}; P{attP,y^{+},w$^{3`}$}*, VDRC# 60100) lines as controls to compare against lines with knockdown of individual homologs. All fly lines were reared on standard yeast

458 *Drosophila* medium at room temperature. All crosses were set and maintained at 25°C, except
459 for the eye knockdown experiments which were maintained at 30°C.
460
461 **Phenotypic analysis of adult wing images**
462 Adult progeny were isolated from crosses between RNAi lines and bx^{MS1096}-*GAL4* driver shortly
463 after eclosion, and kept at 25°C until day 2-5 (**Fig. 2A**). At that point, the progeny were frozen at
464 -80°C, and were then moved to -20°C prior to imaging and storage. Approximately 20-25
465 progeny, both male and female, were collected for each RNAi line tested. The adult wings were
466 plucked from frozen flies and mounted on a glass slide. The slides were covered with a coverslip
467 and sealed using clear nail polish. Adult wing images were captured using a Zeiss Discovery
468 V20 stereoscope (Zeiss, Thornwood, NY, USA), with a ProgRes Speed XT Core 3 camera and
469 CapturePro v.2.8.8 software (Jenoptik AG, Jena, Germany) at 40X magnification.
470 For each non-lethal RNAi line, we scored the adult wing images for five qualitative
471 phenotypes, including wrinkled wing, discoloration, missing veins, ectopic veins, and bristle
472 planar polarity defects, on a scale of 1 (no phenotype) to 5 (lethal) (**Fig. 3C**). Lines showing
473 severely wrinkled wings or lethality were scored as 4 (severe) or 5 (lethal) for all five
474 phenotypes. We calculated the frequency of each phenotypic score (i.e. mild bristle polarity,
475 moderate discoloration) across all of the wing images for each line (**Fig. 3A-B**), and then
476 performed k-means clustering of these values to generate five clusters for overall wing
477 phenotypes (**Fig. 2C**). For quantitative analysis of wing phenotypes, we used the Fiji ImageJ
478 software[100] to calculate the wing area using the Measure Area tool, and calculated the lengths of
479 longitudinal veins L2, L3, L4, and L5 as well as the anterior and posterior crossveins (ACV and
480 PCV), by tracing individual veins using the Segmented Line tool (**Fig. 4A, Supp. Data 2**). We

481 determined discordant homologs when RNAi lines for the same homologs showed inconsistent
482 wing phenotypes. For each homolog with multiple RNAi lines, we checked discordance among
483 RNAi lines for no phenotype versus any qualitative or quantitative phenotypes, followed by
484 discordance for small or large wing measurement phenotypes (**Supp. Data 3**).
485
486 **Phenotypic analysis of adult eye images**
487 We crossed RNAi lines with *GMR-GAL4* to achieve eye-specific knockdown of homologs of
488 CNV and known neurodevelopmental genes. Adult 2-3-day old female progenies from the
489 crosses were collected, immobilized by freezing at -80°C, and then moved to -20°C prior to
490 imaging and storage. Flies were mounted on Blu-tac (Bostik Inc, Wauwatosa, WI, USA) and
491 imaged using an Olympus BX53 compound microscope with LMPLan N 20X air objective using
492 a DP73 c-mount camera at 0.5X magnification (Olympus Corporation, Tokyo, Japan). CellSens
493 Dimension software (Olympus Corporation, Tokyo, Japan) was used to capture the eye images,
494 which were then stacked using the Zerene Stacker software (Zerene Systems LLC, Richland,
495 WA, USA). All eye images presented in this study are maximum projections of 20 consecutive
496 optical z-sections, at a z-step size of 12.1µm. Finally. we used our computational method called
497 *Flynotyper* (https://flynotyper.sourceforge.net) to quantify the degree of rough eye phenotypes
498 present due to knockdown of homologs of CNV or neurodevelopmental genes[60]. *Flynotyper*
499 scores for homologs of 16p11.2 and 3q29, as well as select core neurodevelopmental genes, were
500 derived from our previous studies[41,42,60].
501
502
503

Immunohistochemistry

Wing imaginal discs from third instar larvae were dissected in 1X PBS. The tissues were fixed using 4% paraformaldehyde and blocked using 1% bovine serum albumin (BSA). The wing discs were incubated with primary antibodies using appropriate dilutions overnight at 4°C. We used the following primary antibodies: mouse monoclonal anti-pHistone3 (S10) (1:100 dilutions, Cell Signaling 9706L), rabbit polyclonal anti-cleaved *Drosophila* Dcp1 (Asp216) (1:100 dilutions, Cell Signaling 9578S), mouse monoclonal anti-Wingless (1:200 dilutions, DSHB, 4D4), mouse monoclonal anti-Patched (1:50 dilutions, DSHB, *Drosophila* Ptc/APA1), mouse monoclonal anti-Engrailed (1:50 dilutions, DSHB, 4D9), and mouse monoclonal anti-Delta (1:50 dilutions, DSHB, C594.9B). Following incubation with primary antibodies, the wing discs were washed and incubated with secondary antibodies at 1:200 dilution for two hours at room temperature. We used the following secondary antibodies: Alexa Fluor 647 dye goat anti-mouse (A21235, Molecular Probes by Invitrogen/Life Technologies), Alexa Fluor 568 dye goat anti-rabbit (A11036, Molecular Probes by Invitrogen/Life Technologies), and Alexa Fluor 568 dye goat anti-mouse (A11031, Molecular Probes by Invitrogen/Life Technologies). All washes and antibody dilutions were made using 0.3% PBS with Triton-X.

Third instar larvae wing imaginal discs were mounted in Prolong Gold antifade reagent with DAPI (Thermo Fisher Scientific, P36930) for imaging using an Olympus Fluoview FV1000 laser scanning confocal microscope (Olympus America, Lake Success, NY). Images were acquired using FV10-ASW 2.1 software (Olympus, Waltham, MA, USA). Composite z-stack images were analyzed using the Fiji ImageJ software[100]. To calculate the number of pH3 positive cells within the wing pouch area of the wing discs, we used the AnalyzeParticles function in ImageJ, while manual counting was used to quantify Dcp1 positive cells. We note that cell

proliferation and apoptosis staining for *NCBP2/Cbp20*, *DLG1/dlg1*, *BDH1/CG8888*, and *FBXO45/Fsn* were previously published[42].

Statistical analysis

Significance for the wing area and vein length measurements, cell counts for proliferation and apoptosis, and *Flynotyper* scores were compared to appropriate GD or KK controls using one-tailed or two-tailed Mann-Whitney tests. P-values for each set of experiments were corrected for multiple testing using Benjamini-Hochberg correction. All statistical and clustering analysis was performed using R v.3.6.1 (R Center for Statistical Computing, Vienna, Austria). Details for the statistical tests performed for each dataset are provided in **Supp. Data 10**.

Expression data analysis

We obtained tissue-specific expression data for fly homologs of CNV genes from the FlyAtlas Anatomical Microarray dataset[62]. Raw FPKM (fragments per kilobase of transcript per million reads) expression values for each tissue were categorized as follows: <10, no expression; 10-100, low expression; 100-500, moderate expression; 500-1000, high expression; and >1000, very high expression (**Supp. Data 6**). The median expression among midgut, hindgut, Malpighian tube, and (for adult only) crop tissues was used to represent the overall gut expression. We similarly obtained human tissue-specific expression data for CNV genes from the GTEx Consortium v.1.2 RNA-Seq datasets[64]. Median TPM (transcripts per million reads) expression values for each tissue were categorized as follows: <3, no expression; 3-10, low expression; 10-25, moderate expression; 25-100, high expression; and >100, very high expression (**Supp. Data 6**). The median expression among all brain and heart sub-tissues was used to represent brain and heart

550 expression, while the median expression among all colon, esophagus, small intestine, and
551 stomach sub-tissues was used to represent digestive tract expression. Preferential gene
552 expression for a particular tissue within the GTEx dataset was determined if the expression
553 values for that tissue were greater than the third quartile of all tissue expression values for that
554 gene, plus 1.5 times the interquartile range. Venn diagrams were generated using the Venny
555 webtool (http://bioinfogp.cnb.csic.es/tools/venny) (**Supp. Fig. 3**).
556
557 **Network analysis**
558 We obtained human tissue-specific gene interaction networks for brain, heart, and kidney tissues
559 from the GIANT network database[80] within HumanBase (https://hb.flatironinstitute.org). These
560 networks were built by training a Bayesian classifier based on tissue-specific gene co-expression
561 datasets, which then assigned a posterior probability for interactions between each pair of genes
562 within the genome for a particular tissue. We downloaded the "Top edge" version of each tissue-
563 specific network, and extracted all gene pairs with posterior probabilities >0.2 to create sub-
564 networks containing the top ~0.5% tissue-specific interactions. Next, we identified the shortest
565 paths in each sub-network between human CNV genes whose fly homologs disrupted signaling
566 pathways in the larval wing disc and human genes within each disrupted pathway, using the
567 inverse of the posterior probability as weights for each edge in the network. Gene sets from the
568 human Notch (KEGG:map04330), Wnt (KEGG:map04310) and Hedgehog pathways
569 (KEGG:map04340) were curated from the Kyoto Encyclopedia of Genes and Genomes (KEGG)
570 pathway database[101]. Using the NetworkX Python package[102], we calculated the shortest distance
571 between each CNV gene and pathway gene, and identified connecting genes that were within
572 each of the shortest paths for the three tissue-specific networks. We further tested for enrichment

of Gene Ontology (GO) terms (PantherDB GO-Slim) among the connector genes using the PantherDB Gene List Analysis tool[103]. Lists of the shortest paths and connector genes in each tissue-specific network, as well as enriched GO terms for sets of connector genes, are provided in **Supp. Data 8**. Gene networks were visualized using Cytoscape v.3.7.2[104] using an edge-weighted spring embedded layout.

Mouse and human phenotypic data analysis

Phenotypic data for mouse models of CNV gene homologs, categorized using top-level Mammalian Phenotype Ontology terms, were obtained from the Mouse Genome Informatics (MGI) database[81] (**Supp. Data 4**). Phenotypic data for human carriers of pathogenic CNVs were obtained from the DECIPHER public database[9]. Clinical phenotypes for each CNV carrier were categorized by top-level Human Phenotype Ontology terms[105] using the Orange3 Bioinformatics software library (https://orange-bioinformatics.readthedocs.io), and the frequency of individuals carrying each top-level phenotype term was calculated for each of the ten tested pathogenic CNVs.

DATA AVAILABILITY

All data supporting the findings of this study are available within the paper and its supplementary information files.

CODE AVAILABILITY

All source code for data analysis in this manuscript, including scripts for k-means clustering of fly phenotypes, network connectivity of CNV and developmental pathway genes, and extraction of top-level human phenotype terms, are available on the Girirajan lab GitHub page at https://github.com/girirajanlab/CNV_wing_project.

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CONTRIBUTIONS

T.Y., M.J., S.Y., and S.G. designed the study. T.Y., S.Y., L.P., S.K., D.J.G., A.S., Y.M., J.I., and Z.C.L. performed the functional experiments. T.Y. and M.J. performed the expression and network experiments. T.Y., M.J., and S.G. analyzed the data and wrote the manuscript with input from all authors.

COMPETING OF INTERESTS

The authors declare that they have no competing interests.

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854

855 **FIGURE LEGENDS**

856 **Figure 1. Phenotypic expression of CNV carriers across tissues.** Heatmap with frequencies of
857 non-neuronal developmental phenotypes observed in 1,225 human carriers of 10 pathogenic
858 CNV deletions, curated from the DECIPHER database, is shown. CNV carriers show a variety of
859 phenotypes that manifest across different tissues, including eye, limbs, muscle, and skeleton.
860
861 **Figure 2. Targeted analysis to identify global developmental phenotypes with knockdown**
862 **of homologs of CNV genes. (A)** Strategy for identifying non-neuronal phenotypes and
863 underlying cellular mechanisms for homologs of CNV and known neurodevelopmental genes
864 using the fly wing as a model system. We evaluated 59 *Drosophila* homologs of genes within 10
865 CNV regions and 20 known neurodevelopmental genes (79 total homologs). Using the *UAS-*
866 *GAL4* system with wing-specific bx^{MS1096} driver, we knocked down 136 individual RNAi lines
867 for the CNV and neurodevelopmental homologs, and evaluated qualitative and quantitative
868 phenotypes. We next clustered RNAi lines based on severity of qualitative phenotypes, and
869 compared adult wing phenotypes to phenotypes observed with ubiquitous and eye-specific
870 knockdown of homologs. Furthermore, we evaluated underlying cellular mechanisms for the
871 observed wing-specific phenotypes, and examined the connectivity patterns of candidate
872 homologs for developmental phenotypes in multiple human tissue-specific networks. **(B)**
873 Representative brightfield images of adult wing phenotype severity observed with knockdown of
874 homologs of CNV genes, based on clustering analysis, are shown. **(C)** Heatmap with k-means
875 clustering of qualitative phenotypes in adult female wings across 136 RNAi lines is shown. The
876 color of each cell represents the frequency of individual fly wings (n=20-25 adult wings) for
877 each RNAi line (x-axis) that show a specific severity (no phenotype, mild, moderate, severe,

878 lethal) for the five qualitative phenotypes assessed (y-axis; wrinkled wings, ectopic veins,
879 missing veins, discoloration, bristle planar polarity), as detailed in **Supp. Data 2**. Based on these
880 data, we identified clusters for no phenotype (n=75 lines), mild (n=24 lines), moderate (n=10
881 lines), severe (n=21 lines), and lethal (n=6 lines). **(D)** Summary table of qualitative and
882 quantitative adult wing phenotypes for all tested RNAi lines of homologs of CNV and
883 neurodevelopmental genes. Quantitative phenotype totals do not include lethal RNAi lines for
884 both area and vein length. In addition, L3 vein length totals do not include severe RNAi lines.
885
886 **Figure 3. Qualitative adult wing phenotypes of *Drosophila* homologs of CNV and**
887 **neurodevelopmental genes.** Heatmaps representing the five qualitative adult wing phenotypes
888 for all 136 RNAi lines, with **(A)** all 59 tested homologs for 10 CNV regions and **(B)** 20
889 homologs for neurodevelopmental genes (β-catenin, core neurodevelopmental genes, and
890 microcephaly genes), are shown. The color of each cell represents the frequency of each of the
891 five qualitative phenotypes by severity (wrinkled wings, WR; ectopic veins, EV; missing veins,
892 MV; discoloration, DC; bristle planar polarity, BP), ranging from no phenotype to lethal. **(C)**
893 Representative brightfield images of adult fly wings (scale bar = 500μm) with wing-specific
894 knockdown of homologs of CNV and neurodevelopmental genes showing the five assessed
895 qualitative phenotypes, including discoloration, wrinkled wings, bristle polarity, ectopic veins,
896 and missing veins are shown. The panels in the bx^{MS1096}-*GAL4* control and $C6836^{KK112485}$ images
897 highlight bristle planar polarity phenotypes for the representative images. Black arrowheads
898 highlight ectopic veins and white arrowheads highlight missing veins. Genotypes for the images
899 are: w^{1118}/bx^{MS1096}-*GAL4;+; UAS-Dicer2/+*, w^{1118}/bx^{MS1096}-*GAL4;UAS-Rph*^{GD7330} *RNAi/+;UAS-*
900 *Dicer2/+*, w^{1118}/bx^{MS1096}-*GAL4;UAS-CG15528*^{KK107736} *RNAi/+; UAS-Dicer2/+*, w^{1118}/bx^{MS1096}-

GAL4;UAS-CG6836^{KK112485} RNAi/+; UAS-Dicer2/+, *w^{1118}/bx^{MS1096}-GAL4;+;UAS-CG14182^{GD2738} RNAi/UAS-Dicer2*, and *w^{1118}/bx^{MS1096}-GAL4;UAS-kis^{KK100890} RNAi/+; UAS-Dicer2/+*.

Figure 4. Quantitative adult wing phenotypes of *Drosophila* homologs of CNV and neurodevelopmental genes. (A) Representative brightfield images of adult fly wings (scale bar = 500µm) with wing-specific knockdown of homologs of CNV and neurodevelopmental genes with size defects are shown. The *bx^{MS1096}-GAL4* control image highlights the six veins, including longitudinal veins L2, L3, L4, and L5 as well as the anterior and posterior crossveins (ACV and PCV), that were measured for quantitative analysis. The dotted line in the control image represents the total wing area calculated for each RNAi line. Genotypes for the images are: *w^{1118}/bx^{MS1096}-GAL4;+; UAS-Dicer2/+*, *w^{1118}/bx^{MS1096}-GAL4;UAS-Fmo-2^{KK109203} RNAi/+; UAS-Dicer2/+*, and *w^{1118}/bx^{MS1096}-GAL4;+;UAS-Trpm^{GD4541} RNAi/UAS-Dicer2*. **(B)** Boxplot of L3 vein lengths for knockdown of select homologs in adult fly wings (n = 9-91, *p < 0.05, two-tailed Mann–Whitney test with Benjamini-Hochberg correction). Vein measurements for all other longitudinal veins and crossveins (ACV and PCV) for these lines are represented in **Supp Fig. 2**. **(C)** Boxplot of wing areas for knockdown of select homologs in adult fly wings (n = 9-91, *p < 0.05, two-tailed Mann–Whitney test with Benjamini-Hochberg correction). All boxplots indicate median (center line), 25th and 75th percentiles (bounds of box), and minimum and maximum (whiskers), with red dotted lines representing the control median.

Figure 5. Comparison of wing-specific, eye-specific, and ubiquitous knockdown of homologs of CNV and known neurodevelopmental genes. (A) Heatmap with the penetrance

of phenotypes with ubiquitous knockdown (*da–GAL4*) of select homologs of CNV genes, compared to their adult wing-specific (bx^{MS1096}*–GAL4*) phenotypic severity is shown. **(B)** Boxplots of *Flynotyper*-derived phenotypic scores for adult eyes with eye-specific knockdown (*GMR-GAL4*) of select homologs of CNV and neurodevelopmental genes, normalized as fold-change (FC) to control values ($n = 7–40$, $*p < 0.05$, one-tailed Mann–Whitney test with Benjamini-Hochberg correction). The boxplots are arranged by severity of adult wing phenotypes observed for each RNAi line, while the *Flynotyper* phenotypic scores are categorized into four severity categories: no change (0–1.1 FC), mild (1.1–1.5 FC), moderate (1.5–2.0 FC), and severe (>2.0 FC). **(C)** Boxplot showing the average eye phenotypic scores for 66 RNAi lines of select homologs of CNV and neurodevelopmental genes, normalized as fold-change (FC) to control values, by wing phenotypic category (n=4–30 RNAi lines per group). We did not observe any significant changes in eye phenotype severity across the five wing phenotypic categories (Kruskal-Wallis rank sum test, p=0.567, df = 5, $\chi^2 = 3.881$). Examples of average eye phenotypic scores for RNAi lines with no phenotype ($para^{GD3392\_1}$), mild ($rl^{KK115768}$), and lethal ($dlg1^{GD4689}$) wing phenotype severity are highlighted in the graph. All boxplots indicate median (center line), 25th and 75th percentiles (bounds of box), and minimum and maximum (whiskers), with red dotted lines representing the control median. **(D)** Representative brightfield adult eye (scale bar = 100 µm) and adult wing (scale bar = 500µm) images with tissue-specific knockdown of homologs of CNV genes are shown. Genotypes for the eye images are: w^{1118}*;GMR-GAL4/+; UAS-Dicer2/+,* w^{1118}*;GMR-GAL4/UAS-Lnk*^{KK105731} *RNAi; UAS-Dicer2/+*, w^{1118}*;GMR-GAL4/UAS-mEFTu1*^{GD16961} *RNAi; UAS-Dicer2/+*. Genotypes for the wing images are: w^{1118}/bx^{MS1096}*-GAL4;+; UAS-Dicer2/+*, w^{1118}/bx^{MS1096}*-GAL4; UAS-Lnk*^{KK105731} *RNAi/+; UAS-Dicer2/+*, and w^{1118}/bx^{MS1096}*-GAL4; UAS-mEFTu1*^{GD16961} *RNAi/+; UAS-Dicer2/+*.

Figure 6. Expression patterns of *Drosophila* homologs and human CNV and neurodevelopmental genes across multiple tissues. (A) Heatmap with expression of fly homologs of select CNV and neurodevelopmental genes in multiple *Drosophila* larval and adult tissues, derived from the FlyAtlas Anatomical Microarray dataset, compared with adult wing phenotype severity, is shown. Expression values are grouped into no expression (<10 fragments per kilobase of transcript per million reads, or FPKM), low (10–100 FPKM), moderate (100–500 FPKM), high (500–1000 FPKM), and very high (>1000 FPKM) expression categories. **(B)** Heatmap with expression of select human CNV and neurodevelopmental genes in multiple adult tissues, derived from the Genotype-Tissue Expression (GTEx) dataset v.1.2, is shown. Expression values are grouped into no expression (<3 transcripts per million reads, or TPM), low (3–10 TPM), moderate (10–25 TPM), high (25–100 TPM), and very high (>100 TPM) expression categories. X symbols denote preferential expression in a particular tissue (see Methods). Expression data for all CNV and neurodevelopmental genes are provided in **Supp. Data 6**.

Figure 7. *Drosophila* homologs of CNV and neurodevelopmental genes show altered levels of apoptosis and proliferation. (A) Larval imaginal wing discs (scale bar = 50 µm) stained with nuclear marker DAPI, apoptosis marker dcp1, and cell proliferation marker pH3 illustrate altered levels of apoptosis and cell proliferation due to wing-specific knockdown of select fly homologs of CNV genes. We quantified the number of stained cells within the wing pouch of the wing disc (white box), which becomes the adult wing. Additional representative images of select homologs are presented in **Supp Fig. 5**. Genotypes for the wing images are: w^{1118}/bx^{MS1096}-*GAL4;+; UAS-Dicer2/+*, w^{1118}/bx^{MS1096}-*GAL4;+; UAS-Aatf*^{GD7229} *RNAi/UAS-Dicer2*, w^{1118}/bx^{MS1096}-*GAL4;UAS-*

970 *Pp4-19C*^{GD9561}*/+; UAS-Dicer2/+,* w^{1118}*/bx*^{MS1096}*-GAL4;+; UAS-Atx2*^{GD11562} *RNAi/UAS-Dicer2*,
971 and w^{1118}*/bx*^{MS1096}*-GAL4;+; UAS-Sin*^{GD7027} *RNAi/UAS-Dicer2.* **(B)** Box plot of dcp1-positive
972 cells in larval wing discs with knockdown of select fly homologs of CNV and
973 neurodevelopmental genes, normalized to controls ($n = 7–18$, $*p < 0.05$, two-tailed Mann–
974 Whitney test with Benjamini-Hochberg correction). We note that several RNAi lines showed
975 severe dcp1 staining across the entire wing disc and could not be quantified. The number of dcp1
976 positive cells were calculated manually. **(C)** Box plot of pH3-positive cells in the larval wing
977 discs with knockdown of select fly homologs of CNV and neurodevelopmental genes,
978 normalized to controls ($n = 6–18$, $*p < 0.05$, two-tailed Mann–Whitney test with Benjamini-
979 Hochberg correction). The number of pH3 positive cells were calculated using the
980 AnalyzeParticles function in ImageJ. All boxplots indicate median (center line), 25th and 75th
981 percentiles (bounds of box), and minimum and maximum (whiskers), with red dotted lines
982 representing the control median.
983
984 **Figure 8. Candidate *Drosophila* homologs of genes within CNV regions interact with**
985 **conserved signaling pathways.** Larval imaginal wing discs (scale bar = 50 μm) stained with **(A)**
986 wingless, **(B)** patched, **(C)** engrailed, and **(D)** delta illustrate disrupted expression patterns for
987 proteins located within the Wnt (wingless), Hedgehog (patched and engrailed), and Notch (delta)
988 signaling pathways due to wing-specific knockdown of select fly homologs of CNV and
989 neurodevelopmental genes. Dotted yellow boxes represent expected expression patterns for
990 signaling proteins in *bx*^{MS1096}*-GAL4* control images. White arrowheads and dotted white boxes
991 highlight disruptions in expression patterns of signaling proteins with knockdown of CNV or
992 neurodevelopmental genes. Additional representative images of select homologs are presented in

Supp Fig. 7. Genotypes for the wing images are: *w^{1118}/bx^{MS1096}-GAL4;+; UAS-Dicer2/+*, *w^{1118}/bx^{MS1096}-GAL4;UAS-Pp4-19C^{GD9561}/+; UAS-Dicer2/+*, *w^{1118}/bx^{MS1096}-GAL4;UAS-Cbp20^{KK109448}/+; UAS-Dicer2/+*, *w^{1118}/bx^{MS1096}-GAL4;+; UAS-Sin^{GD7027} RNAi/UAS-Dicer2*, *w^{1118}/bx^{MS1096}-GAL4;+; UAS-Aatf^{GD7229} RNAi/UAS-Dicer2*, and *w^{1118}/bx^{MS1096}-GAL4;UAS-Klp61F^{GD14149}/+; UAS-Dicer2/+*.

Figure 9. Connectivity of human CNV genes with conserved signaling pathway genes in human tissue-specific networks. (A) Representative diagrams of eight human CNV and neurodevelopmental genes whose fly homologs disrupt the Notch signaling pathway and 57 human Notch signaling genes within kidney, heart, and brain-specific gene interaction networks are shown. Yellow nodes represent CNV and neurodevelopmental genes, pink nodes represent Notch signaling pathway genes, and green nodes represent connector genes within the shortest paths between CNV and Notch pathway genes. **(B)** Violin plots showing the average connectivity (i.e. inverse of shortest path lengths) of CNV genes to genes in Hedgehog, Notch, and Wnt signaling pathways across the tested tissue-specific networks (n=322–810 pairwise interactions, *$p < 0.05$, two-tailed Welch's t-test with Benjamini-Hochberg correction). **(C)** Table showing enriched clusters of Gene Ontology (GO) Biological Process terms for connector genes observed for each signaling pathway in the three tested tissue-specific networks, categorized by enrichments in ubiquitous, neuronal, and non-neuronal tissues ($p<0.05$, Fisher's Exact test with Benjamini-Hochberg correction).

Figure 10. A multigenic model for neuronal and non-neuronal phenotypes associated with pathogenic CNVs. Schematic of a multigenic model for neuronal and non-neuronal phenotypes

1016 associated with pathogenic CNVs. While a subset of genes within CNV regions contribute
1017 towards tissue-specific phenotypes, a majority of genes contribute towards both neuronal and
1018 non-neuronal phenotypes through disruption of developmental signaling pathways and global
1019 biological processes.

Figure 1

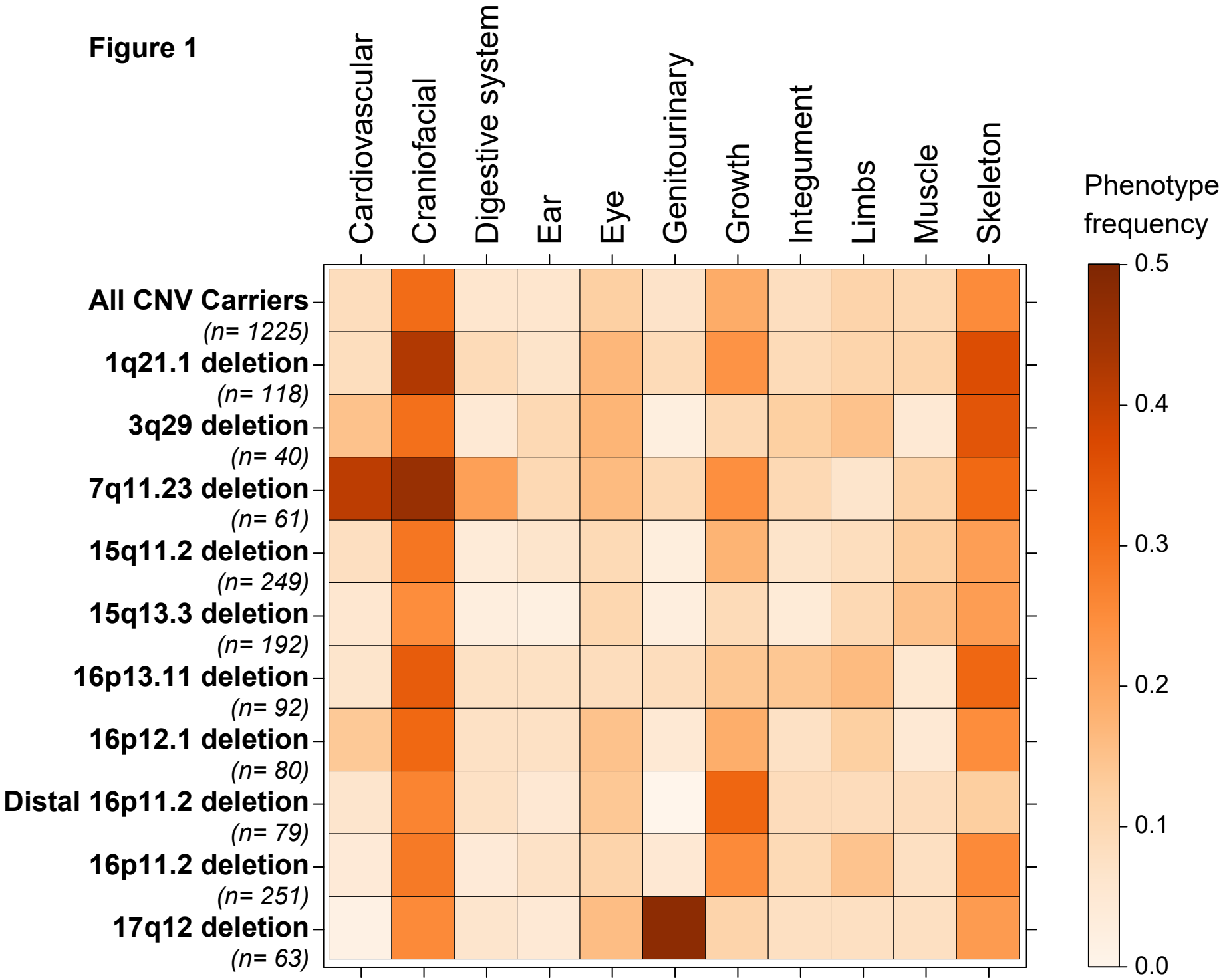

Cardiovascular
Craniofacial
Digestive system
Ear
Eye
Genitourinary
Growth
Integument
Limbs
Muscle
Skeleton
All CNV Carriers (n= 1225)
1q21.1 deletion (n= 118)
3q29 deletion (n= 40)
7q11.23 deletion (n= 61)
15q11.2 deletion (n= 249)
15q13.3 deletion (n= 192)
16p13.11 deletion (n= 92)
16p12.1 deletion (n= 80)
Distal 16p11.2 deletion (n= 79)
16p11.2 deletion (n= 251)
17q12 deletion (n= 63)
Phenotype frequency
0.5
0.4
0.3
0.2
0.1
0.0

Figure 2

A Experimental strategy

Wing-specific driver *bx^{MS1096}-GAL4* × ***UAS-RNAi*** (79 genes)

Phenotyping of adult wings

Qualitative analysis
- Wrinkled wings
- Ectopic veins
- Missing veins
- Discoloration
- Bristle planar polarity

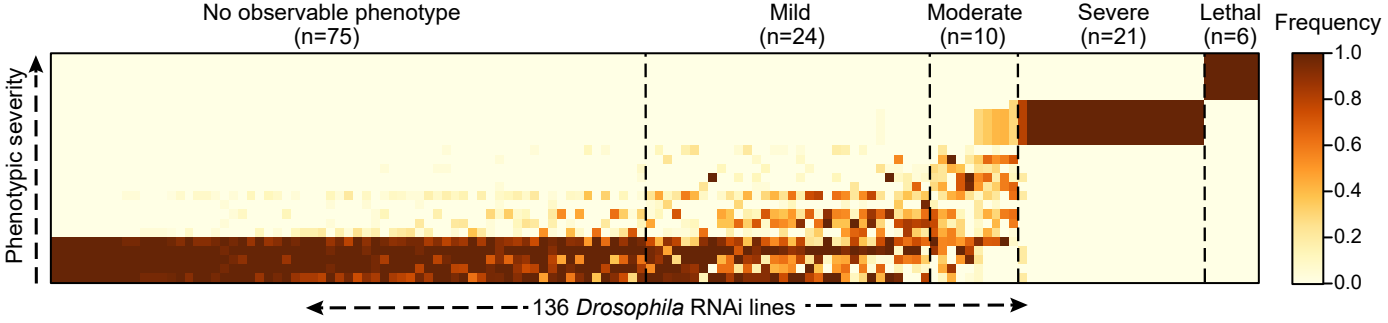
Quantitative analysis
- Longitudinal veins (L2,L3,L4,L5)
- Crossveins (ACV, PCV)
- Wing area

Tissue-specific effects
- Compare to ubiquitous knockdown
- Compare to eye-specific knockdown
- Gene expression across multiple tissues (Human and flies)

Cellular processes and developmental pathways
- Apoptosis
- Cell proliferation
- Disruptions in signaling pathways
- Human tissue-specific network analysis (Brain, heart, kidney)

B Phenotypic analysis of adult wings in *Drosophila*

C K-means clustering of qualitative wing data



D Summary of qualitative and quantitative adult wing phenotypes

Fly homologs	RNAi lines	Qualitative phenotypes					Quantitative phenotypes	
		No pheno.	Mild	Moderate	Severe	Lethal	Wing area	L3 vein length
CNV genes (15q11.2, 15q13.3, 16p11.2, Distal 16p11.2, 16p12.1, 16p13.11, 17q12, 1q21.1, 3q29, 7q11.23)	95	45	21	7	16	6	65	53
Known neurodevelopmental genes (β-catenin, core genes, microcephaly)	41	30	3	3	5	0	24	21
Total	136	75	24	10	21	6	89	74

Figure 3

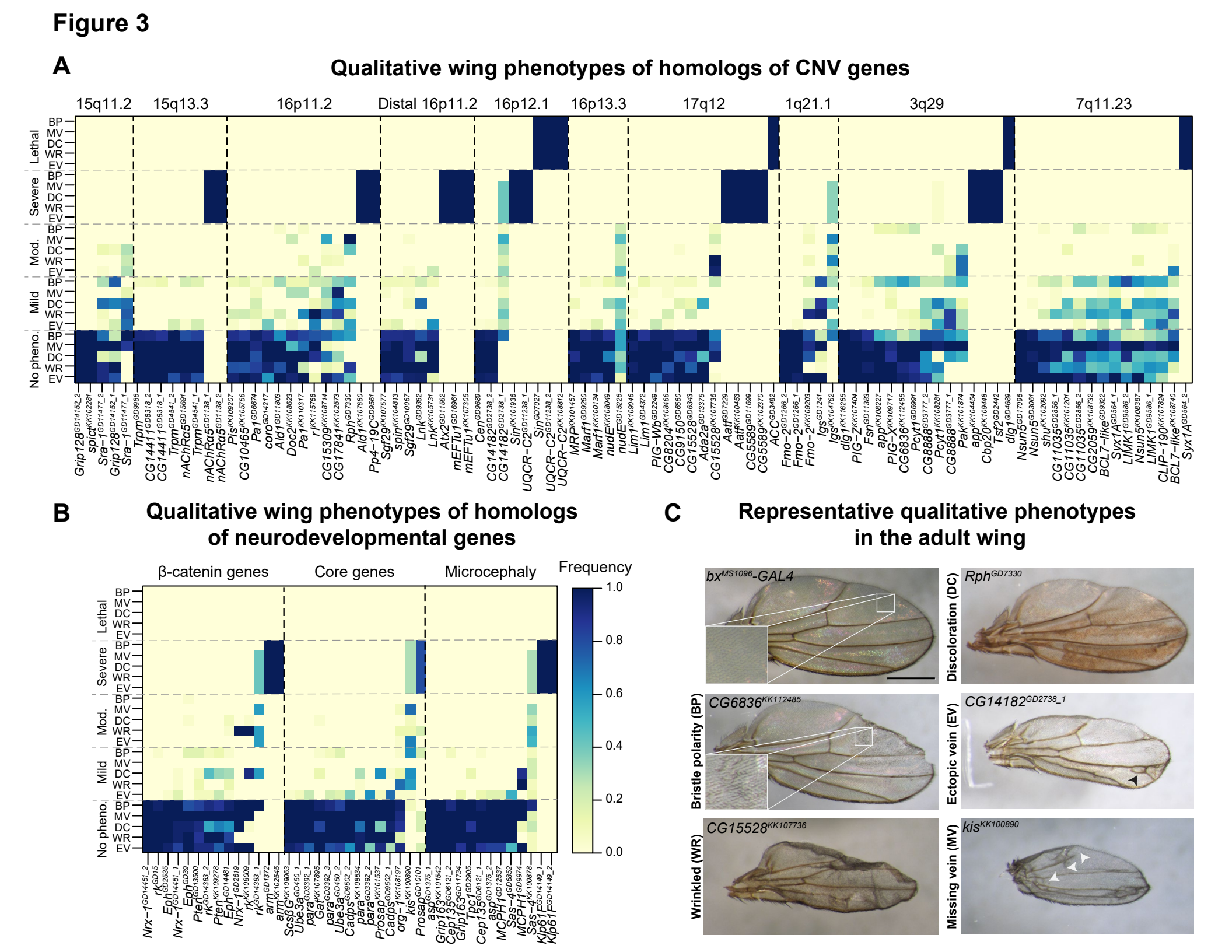

A
Qualitative wing phenotypes of homologs of CNV genes
15q11.2
15q13.3
16p11.2
Distal 16p11.2
16p12.1
16p13.3
17q12
1q21.1
3q29
7q11.23
Lethal
Severe
Mod.
Mild
No pheno.
BP
MV
DC
WR
EV
B
Qualitative wing phenotypes of homologs of neurodevelopmental genes
β-catenin genes
Core genes
Microcephaly
Frequency
1.0
0.8
0.6
0.4
0.2
0.0
C
Representative qualitative phenotypes in the adult wing
bx^MS1096-GAL4
Rph^GD7330
Discoloration (DC)
CG6836^KK112485
Bristle polarity (BP)
CG14182^GD2738_1
Ectopic vein (EV)
CG15528^KK107736
Wrinkled (WR)
kis^KK100890
Missing vein (MV)

Figure 4

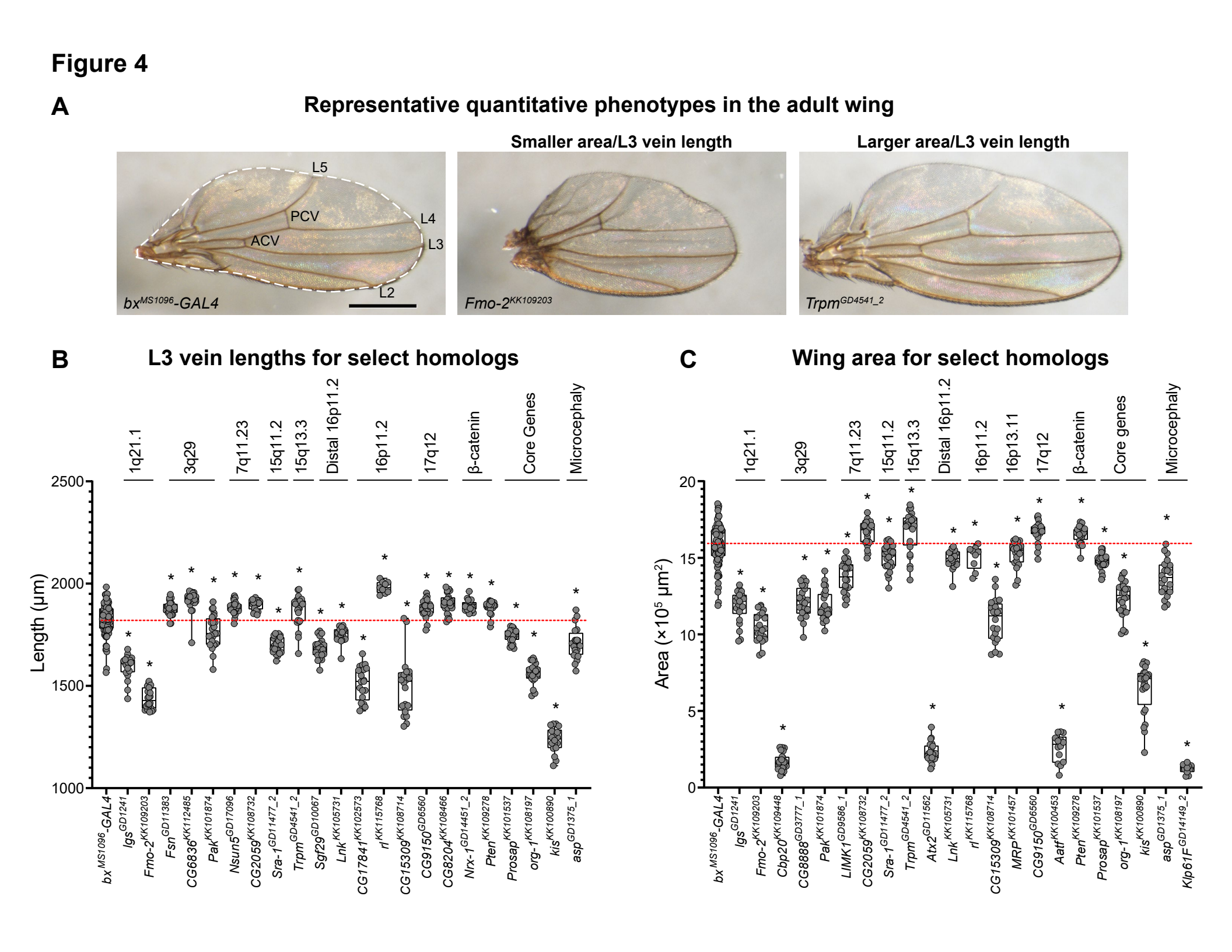

A
Representative quantitative phenotypes in the adult wing
Smaller area/L3 vein length
Larger area/L3 vein length
L5
PCV
ACV
L4
L3
L2
bx^MS1096-GAL4
Fmo-2^KK109203
Trpm^GD4541_2
B
L3 vein lengths for select homologs
C
Wing area for select homologs
1q21.1
3q29
7q11.23
15q11.2
15q13.3
Distal 16p11.2
16p11.2
17q12
β-catenin
Core Genes
Microcephaly
16p13.11
Core genes
Length (µm)
Area (×10^5 µm^2)
bx^MS1096-GAL4
lgs^GD1241
Fmo-2^KK109203
Fsn^GD11383
CG6836^KK112485
Pak^KK101874
Nsun5^GD17096
CG2059^KK108732
Sra-1^GD11477_2
Trpm^GD4541_2
Sgf29^GD10067
Lnk^KK105731
CG17841^KK102573
rl^KK115768
CG15309^KK108714
CG9150^GD6560
CG8204^KK108466
Nrx-1^GD14451_2
Pten^KK109278
Prosap^KK101537
org-1^KK108197
kis^KK100890
asp^GD1375_1
Cbp20^KK109448
CG8888^GD3777_1
LIMK1^GD9586_1
Atx2^GD11562
MRP^KK101457
Aatf^KK100453
Klp61F^GD14149_2

Figure 5

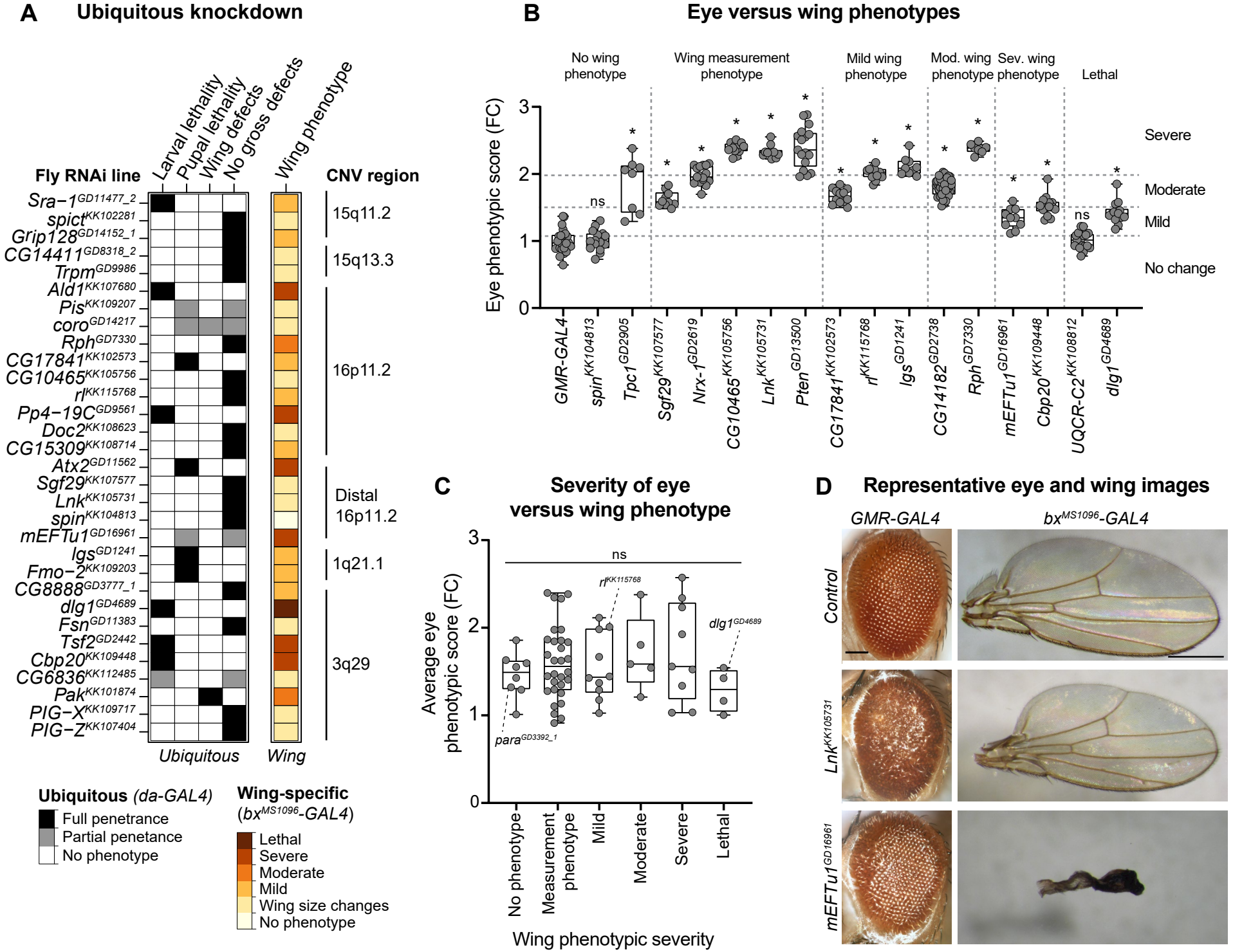

A
Ubiquitous knockdown
Larval lethality
Pupal lethality
Wing defects
No gross defects
Wing phenotype
Fly RNAi line
CNV region
Sra−1GD11477_2
spictKK102281
Grip128GD14152_1
CG14411GD8318_2
TrpmGD9986
Ald1KK107680
PisKK109207
coroGD14217
RphGD7330
CG17841KK102573
CG10465KK105756
rlKK115768
Pp4−19CGD9561
Doc2KK108623
CG15309KK108714
Atx2GD11562
Sgf29KK107577
LnkKK105731
spinKK104813
mEFTu1GD16961
lgsGD1241
Fmo−2KK109203
CG8888GD3777_1
dlg1GD4689
FsnGD11383
Tsf2GD2442
Cbp20KK109448
CG6836KK112485
PakKK101874
PIG−XKK109717
PIG−ZKK107404
15q11.2
15q13.3
16p11.2
Distal 16p11.2
1q21.1
3q29
Ubiquitous
Wing
Ubiquitous (da-GAL4)
Full penetrance
Partial penetance
No phenotype
Wing-specific (bxMS1096-GAL4)
Lethal
Severe
Moderate
Mild
Wing size changes
No phenotype
B
Eye versus wing phenotypes
No wing phenotype
Wing measurement phenotype
Mild wing phenotype
Mod. wing phenotype
Sev. wing phenotype
Lethal
Eye phenotypic score (FC)
Severe
Moderate
Mild
No change
ns
GMR-GAL4
spinKK104813
Tpc1GD2905
Sgf29KK107577
Nrx-1GD2619
CG10465KK105756
LnkKK105731
PtenGD13500
CG17841KK102573
rlKK115768
lgsGD1241
CG14182GD2738
RphGD7330
mEFTu1GD16961
Cbp20KK109448
UQCR-C2KK108812
dlg1GD4689
C
Severity of eye versus wing phenotype
Average eye phenotypic score (FC)
ns
rlKK115768
dlg1GD4689
paraGD3392_1
No phenotype
Measurement phenotype
Mild
Moderate
Severe
Lethal
Wing phenotypic severity
D
Representative eye and wing images
GMR-GAL4
bxMS1096-GAL4
Control
LnkKK105731
mEFTu1GD16961

Figure 6

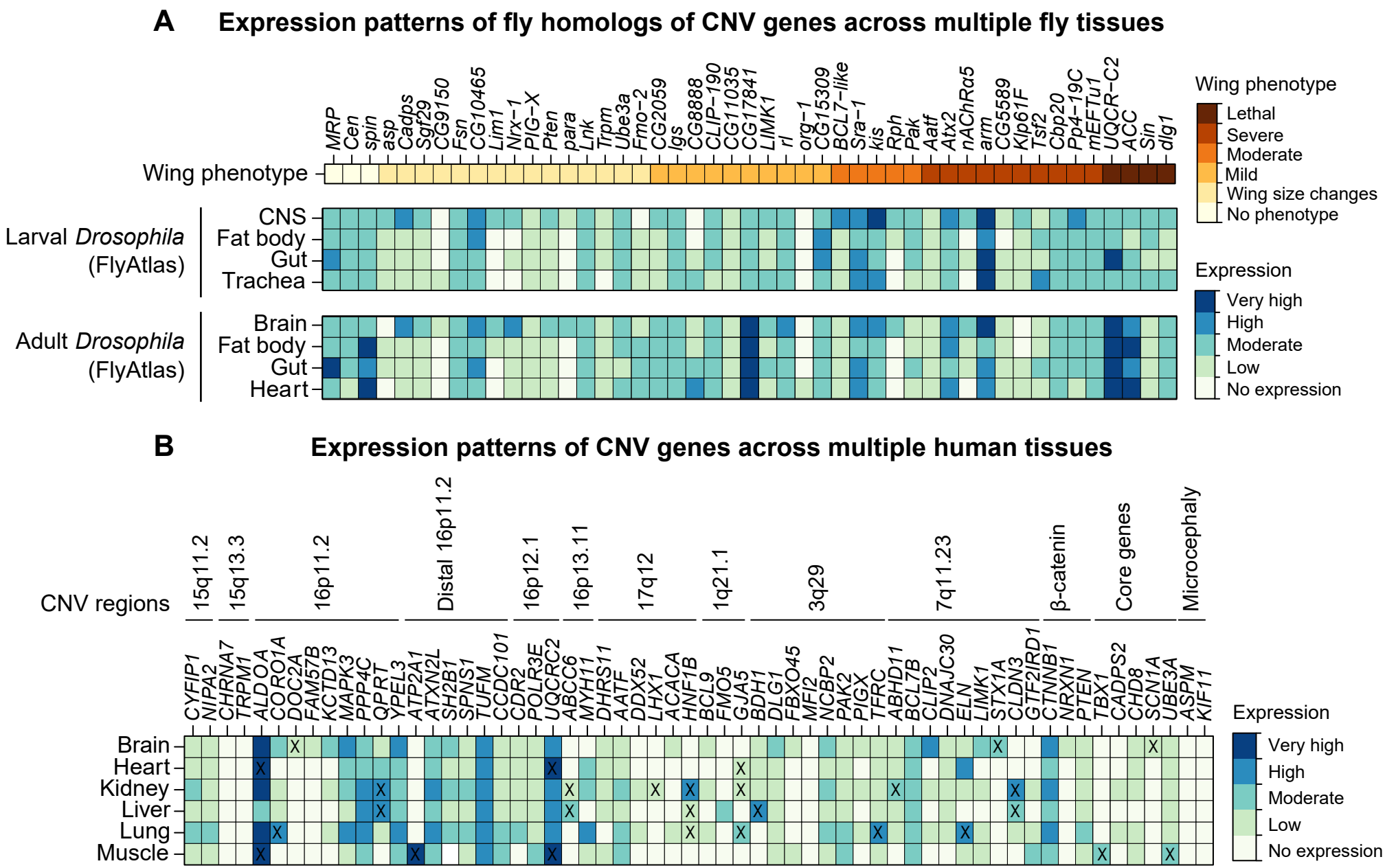

A
Expression patterns of fly homologs of CNV genes across multiple fly tissues
MRP Cen spin asp Cadps Sgf29 CG9150 Fsn CG10465 Lim1 Nrx−1 PIG−X Pten para Lnk Trpm Ube3a Fmo−2 CG2059 Igs CG8888 CLIP−190 CG11035 CG17841 LIMK1 rl org−1 CG15309 BCL7−like Sra−1 kis Rph Pak Aatf Atx2 nAChRα5 arm CG5589 Klp61F Tsf2 Cbp20 Pp4−19C mEFTu1 UQCR−C2 ACC Sin dlg1
Wing phenotype
Larval Drosophila (FlyAtlas)
CNS
Fat body
Gut
Trachea
Adult Drosophila (FlyAtlas)
Brain
Fat body
Gut
Heart
Wing phenotype
Lethal
Severe
Moderate
Mild
Wing size changes
No phenotype
Expression
Very high
High
Moderate
Low
No expression
B
Expression patterns of CNV genes across multiple human tissues
CNV regions
15q11.2
15q13.3
16p11.2
Distal 16p11.2
16p12.1
16p13.11
17q12
1q21.1
3q29
7q11.23
β-catenin
Core genes
Microcephaly
CYFIP1 NIPA2 CHRNA7 TRPM1 ALDOA CORO1A DOC2A FAM57B KCTD13 MAPK3 PPP4C QPRT YPEL3 ATP2A1 ATXN2L SH2B1 SPNS1 TUFM CCDC101 CDR2 POLR3E UQCRC2 ABCC6 MYH11 DHRS11 AATF DDX52 LHX1 ACACA HNF1B BCL9 FMO5 GJA5 BDH1 DLG1 FBXO45 MFI2 NCBP2 PAK2 PIGX TFRC ABHD11 BCL7B CLIP2 DNAJC30 ELN LIMK1 STX1A CLDN3 GTF2IRD1 CTNNB1 NRXN1 PTEN TBX1 CADPS2 CHD8 SCN1A UBE3A ASPM KIF11
Brain
Heart
Kidney
Liver
Lung
Muscle
Expression
Very high
High
Moderate
Low
No expression

Figure 7

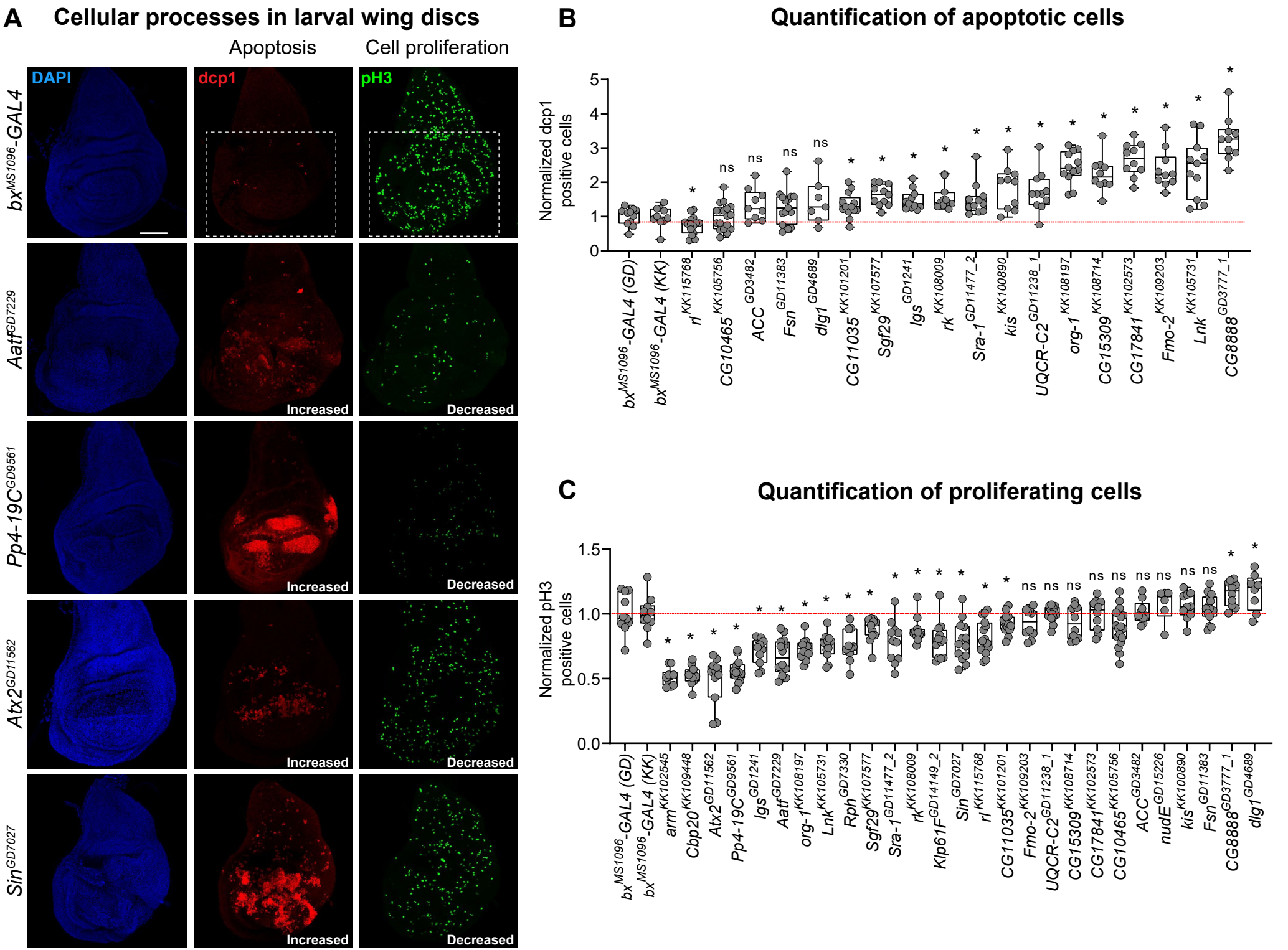

A
Cellular processes in larval wing discs
Apoptosis
Cell proliferation
DAPI
dcp1
pH3
bx^MS1096-GAL4
Aatf^GD7229
Pp4-19C^GD9561
Atx2^GD11562
Sin^GD7027
Increased
Decreased
B
Quantification of apoptotic cells
Normalized dcp1 positive cells
bx^MS1096-GAL4 (GD)
bx^MS1096-GAL4 (KK)
rl^KK115768
CG10465^KK105756
ACC^GD3482
Fsn^GD11383
dlg1^GD4689
CG11035^KK101201
Sgf29^KK107577
lgs^GD1241
rk^KK108009
Sra-1^GD11477_2
kis^KK100890
UQCR-C2^GD11238_1
org-1^KK108197
CG15309^KK108714
CG17841^KK102573
Fmo-2^KK109203
Lnk^KK105731
CG8888^GD3777_1
C
Quantification of proliferating cells
Normalized pH3 positive cells
arm^KK102545
Cbp20^KK109448
Atx2^GD11562
Pp4-19C^GD9561
Aatf^GD7229
Rph^GD7330
Klp61F^GD14149_2
Sin^GD7027
nudE^GD15226
Fsn^GD11383
dlg1^GD4689
ns

Figure 8

Disruption of signaling pathways in larval wing discs

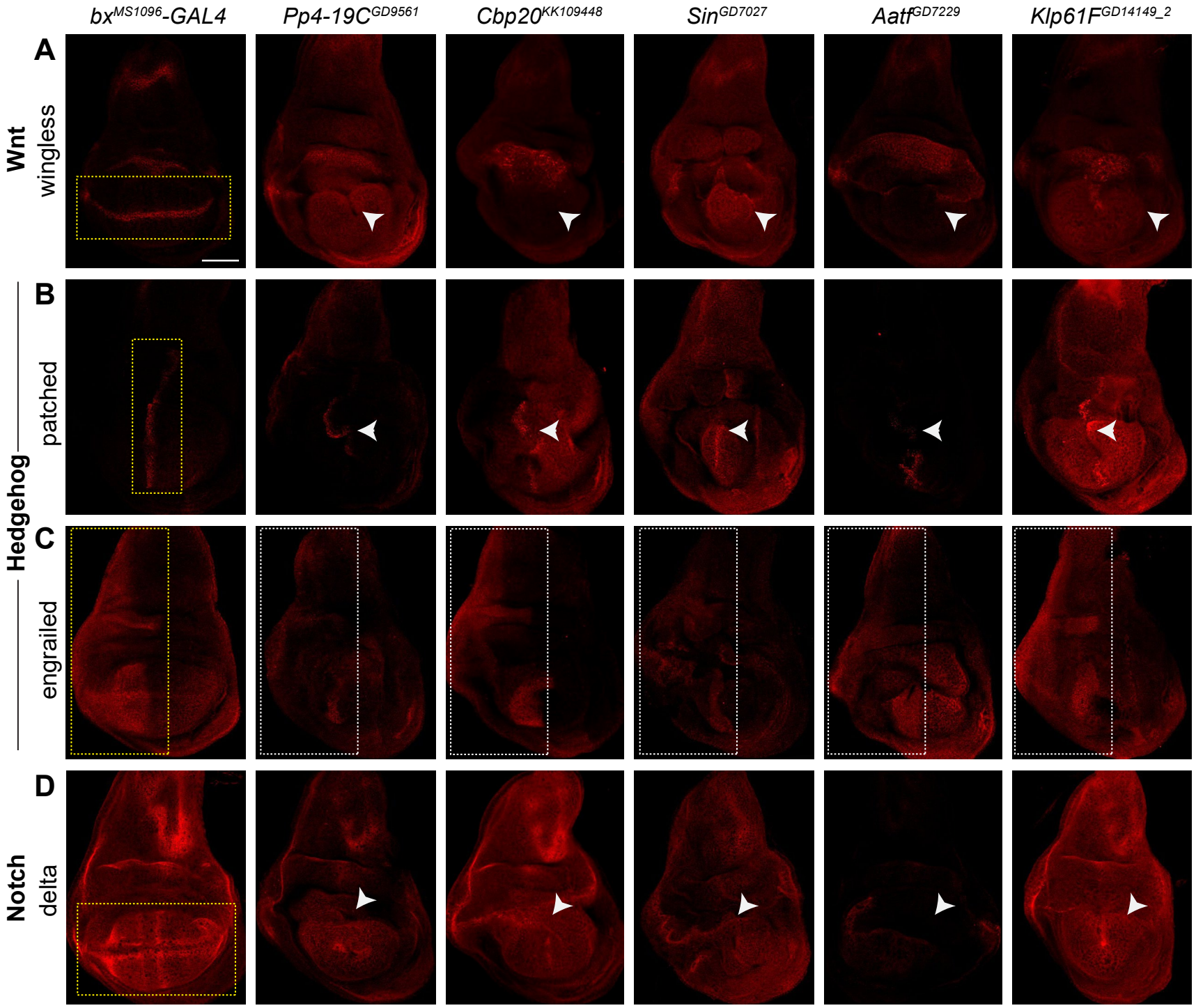
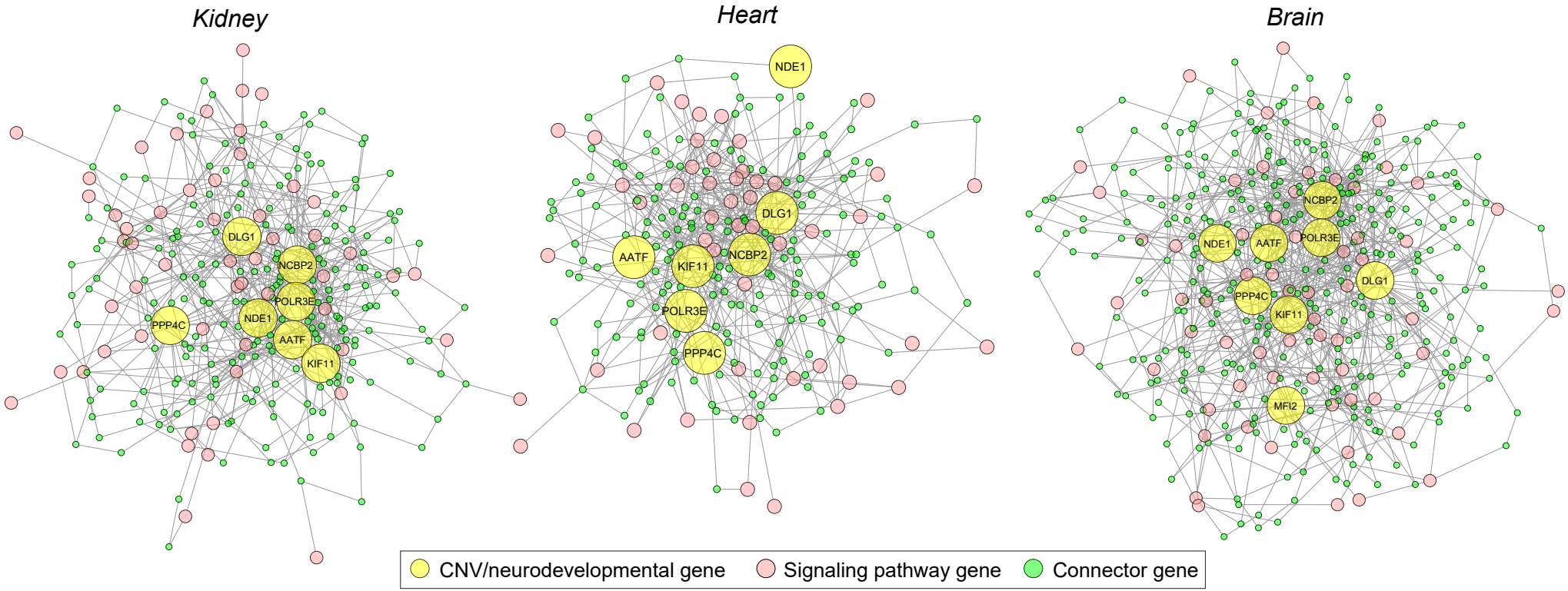
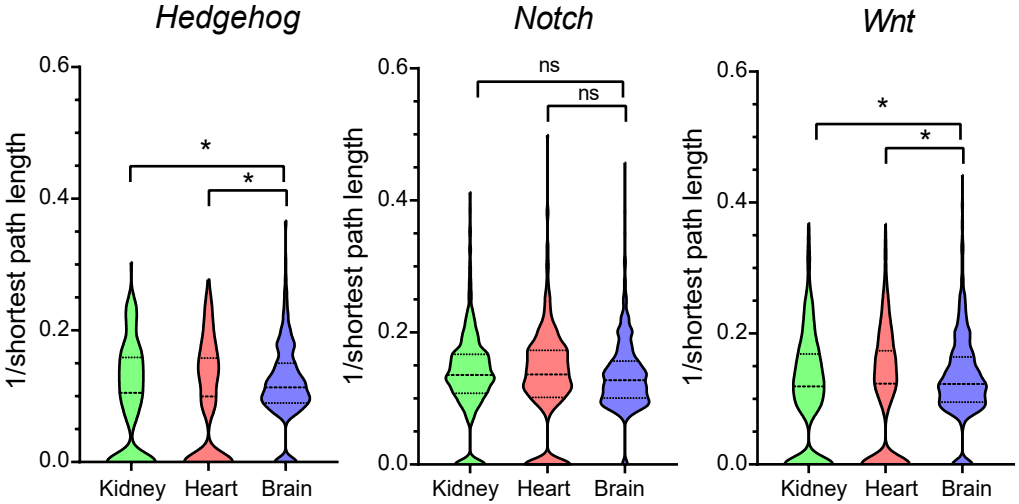


Figure 9

A Human CNV genes interact with Notch signaling pathway genes in multiple tissues



B Average connectivity of CNV genes to genes in signaling pathways



C GO term enrichment for connector genes in tissue-specific networks

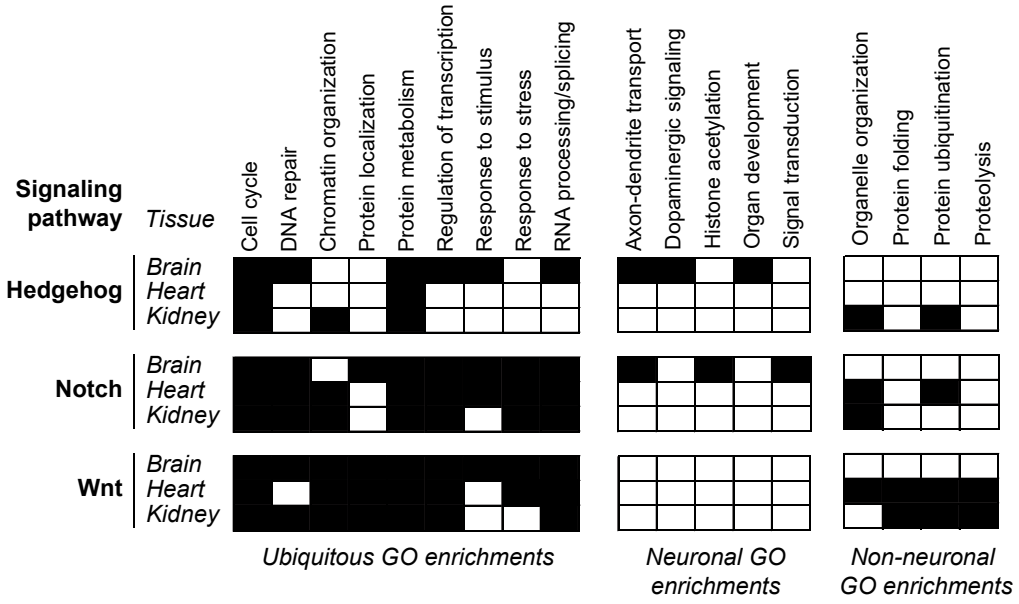
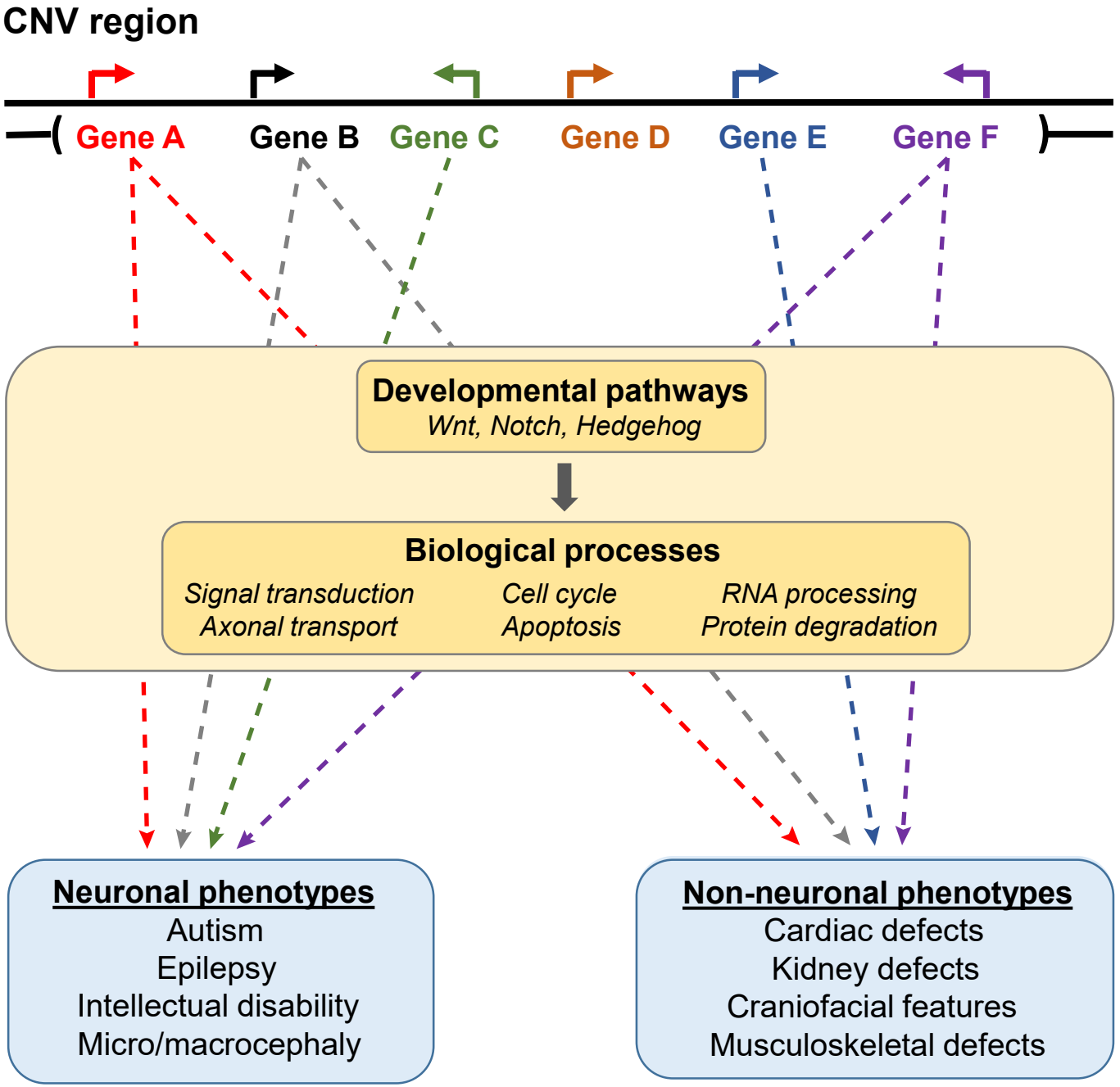


Figure 10


CNV region
Gene A
Gene B
Gene C
Gene D
Gene E
Gene F
Developmental pathways
Wnt, Notch, Hedgehog
Biological processes
Signal transduction
Axonal transport
Cell cycle
Apoptosis
RNA processing
Protein degradation
Neuronal phenotypes
Autism
Epilepsy
Intellectual disability
Micro/macrocephaly
Non-neuronal phenotypes
Cardiac defects
Kidney defects
Craniofacial features
Musculoskeletal defects