

GENERAL ARTICLE

Transcriptional dysregulation in developing trigeminal sensory neurons in the *LgDel* mouse model of DiGeorge 22q11.2 deletion syndrome

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Abstract

LgDel mice, which model the heterozygous deletion of genes at human chromosome 22q11.2 associated with DiGeorge/22q11.2 deletion syndrome (22q11DS), have cranial nerve and craniofacial dysfunction as well as disrupted suckling, feeding and swallowing, similar to key 22q11DS phenotypes. Divergent trigeminal nerve (CN V) differentiation and altered trigeminal ganglion (CNgV) cellular composition prefigure these disruptions in *LgDel* embryos. We therefore asked whether a distinct transcriptional state in a specific population of early differentiating *LgDel* cranial sensory neurons, those in CNgV, a major source of innervation for appropriate oropharyngeal function, underlies this departure from typical development. *LgDel* versus wild-type (WT) CNgV transcriptomes differ significantly at E10.5 just after the ganglion has coalesced. Some changes parallel altered proportions of cranial placode versus cranial neural crest-derived CNgV cells. Others are consistent with a shift in anterior–posterior patterning associated with divergent *LgDel* cranial nerve differentiation. The most robust quantitative distinction, however, is statistically verifiable increased variability of expression levels for most of the over 17 000 genes expressed in common in *LgDel* versus WT CNgV. Thus, quantitative expression changes of functionally relevant genes and increased stochastic variation across the entire CNgV transcriptome at the onset of CN V differentiation prefigure subsequent disruption of cranial nerve differentiation and oropharyngeal function in *LgDel* mice.

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Introduction

The potential relationship between transcriptional differences, anomalous neural circuit development and behavioral deficits has been explored for a number of neurodevelopmental and psychiatric disorders in older children and adults (1). Less attention, however, has been given to transcriptional distinctions that arise early in embryogenesis, perhaps modifying circuits for behaviors that must be in place by birth to ensure survival, growth and health. These behaviors include suckling, feeding and swallowing (S/F/S), whose disruption—perinatal dysphagia—results in substantial health challenges from birth onward, especially in infants and children with a broad range of developmental syndromes (2). We found that S/F/S is compromised in the *LgDel* mouse model of DiGeorge/22q11.2 deletion syndrome (22q11DS), a common genetic disorder (1/3000 live births) (3–5) with an enhanced incidence of perinatal dysphagia (5–7). We now ask whether transcriptional divergence during initial differentiation of key neurons and precursors that facilitate optimal S/F/S—those in the trigeminal ganglion (CNgV) (8) prefigures altered cranial nerve development and function in *LgDel* mice.

CNgV provides sensory innervation to the face, lips, oral cavity and anterior tongue critical for initiation of S/F/S and thus must develop appropriately prior to birth (5). CNgV, like most cranial ganglia, consists of mechanosensory neurons derived from neurogenic cranial placodes and nociceptive neurons derived from hindbrain neural crest (9). Our previous observations (6) show that patterning of the hindbrain neural crest, which gives rise to CNgV nociceptive neurons, is disrupted by the presumed 50% decrease in 22q11 gene dosage by E10.5. This disruption, as well as expression of several 22q11 orthologues in CNgV, and potential for altered interactions between distinct cell classes within and surrounding the ganglion (6,10), all suggest that the *LgDel* CNgV transcriptional state may differ from wild-type (WT). Such early differences in *LgDel*, and potentially in 22q11DS, may ultimately compromise sensory neuron identity and/or disrupt sensory innervation and function, resulting in sub-optimal CNgV-related oropharyngeal behaviors including dysphagia. Accordingly, we used RNA-Seq to define transcriptomes of microdissected *LgDel* and WT CNgV at embryonic day (E) 10.5, just after the ganglion coalesces (6,11), sensory neurogenesis accelerates and axons begin to grow. We analyzed multiple, pooled biological replicates from each genotype (12) to minimize contributions of individual variability, eliminate statistical ‘noise’ and securely identify expression differences due to 22q11 gene deletion with appropriate statistical power in a critical population of cranial sensory neurons.

We found that *LgDel* and WT E10.5 CNgV transcriptomes differ in parallel with differences in CNgV cellular composition in the two genotypes. These differences are distinct from those in transcriptomes of E10.5 *LgDel* or WT whole embryos, emphasizing the value of microdissection to accurately define transcriptional landscapes of developmentally critical cell populations. Differential expression levels of genes that may regulate neuronal differentiation suggest potential mechanisms that can lead to disrupted cranial nerve differentiation and function in *LgDel* mouse pups. The most striking quantitative distinction, however, is statistically verifiable increased expression variability across the entire *LgDel* CNgV transcriptome. Apparently, a stochastically variable transcriptome is a hallmark of *LgDel* cranial sensory neurons at the embryonic stage when cranial neural circuit development essential for optimal S/F/S diverges from the WT differentiation program.

Results

Developmental phenotypes suggest divergent transcriptomes for WT versus *LgDel* CNgV

We showed previously that CN V is dysmorphic in *LgDel* E10.5 embryos compared to WT (6). To visualize CNgV itself, we immunostained whole E10.5 embryos for β III-tubulin, which detects early differentiating neurons as well as axons (Fig. 1A and B) (13). CNgV is dysmorphic in *LgDel* E10.5 embryos; its size and shape are altered, and the extension of axon fascicles from all three divisions (ophthalmic: op; maxillary: mx; mandibular: md; Fig. 1A and B, and inset) is aberrant. To determine if CNgV cellular composition changes similarly at E10.5, we quantified presumed placode derived (Six1 immunolabeled) and neural crest (Wnt1:Cre recombined, eGFP reporter-labeled) associated populations (Fig. 1C and D) (11). There is a decreased proportion of Wnt1:Cre/eGFP labeled cells in *LgDel* CNgV (29% Wnt1:Cre/eGFP in *LgDel*; 35% Wnt1:Cre/eGFP in WT; $P < 0.02$), as well as a corresponding increase in the proportion of Six1 labeled cells (34% Six1 in *LgDel*; 26% Six1 in WT; $P < 0.02$; Fig. 1E). Moreover, a non-Wnt1:Cre recombined, non-Six1 expressing, DAPI-labeled population, associated with Foxd3-expressing neural crest (11), also declines, similar to the Wnt1:Cre/eGFP population (21% in *LgDel*; 29% in WT; $P < 0.04$). The developmental origin of these changes in CNgV may reflect interactions with surrounding neural crest-derived mesenchyme (14,15), which in *LgDel* embryos may also be distinct due to its origin from ‘posteriorized’ hindbrain rhombomeres (see below) or hindbrain target cells as axons extend centrally. Nevertheless, CNgV differentiation diverges in *LgDel* versus WT by the time the ganglion has fully coalesced at E10.5.

LgDel and WT CNgV transcriptome differences

Distinctions between *LgDel* and WT CNgV cellular composition suggest that altered 22q11.2 gene dosage due to heterozygous deletion may result in divergent transcriptional states at the earliest stages of gangliogenesis, leading to anomalous CNgV cell identity and differentiation. CNgV begins to form via local delamination and translocation of cranial ectodermal placode cells as well as arrival and accumulation of a small number of neural crest cells around E9.5 in the mouse; however, it is not fully defined cytologically until E10.5 (11). Thus, we microdissected and pooled CNgV from *LgDel* and WT E10.5 embryos and then sequenced and analyzed transcriptomes ($n = 5$ CNgV pools/genotype, 6 ganglia/pool from at least 3 different embryos/3 different litters; 1 pool = 1 biological replicate), the earliest stage when the ganglion can be confidently identified and isolated accurately from the whole embryo (Fig. 2A and B).

This analysis of early developing WT versus *LgDel* CNgV sensory neurons and precursors identified 17 190 unique genes with non-zero FPKM values shared by both genotypes (Supplemental Table 2). There are 17 199 non-zero reads in WT and 17 221 non-zero reads in *LgDel*. Equally robust detection of similar transcripts in both genotypes indicates that E10.5 *LgDel* CNgV cells are transcriptionally active at levels approximating WT. Analysis of *LgDel* CNgV versus WT showed similar number of up- and downregulated genes (8677 versus 8553; Fig. 2B). From the total set of expressed CNgV genes, we found 134 genes differentially expressed at FDR $q < 0.1$ in *LgDel* versus WT CNgV (Fig. 2C and Supplemental Table 2). Of these 134 genes, 77 are upregulated and 57 downregulated in *LgDel* versus WT CNgV. The murine orthologues of the 22q11-deleted genes should be among the 134 differentially expressed genes, detected at an

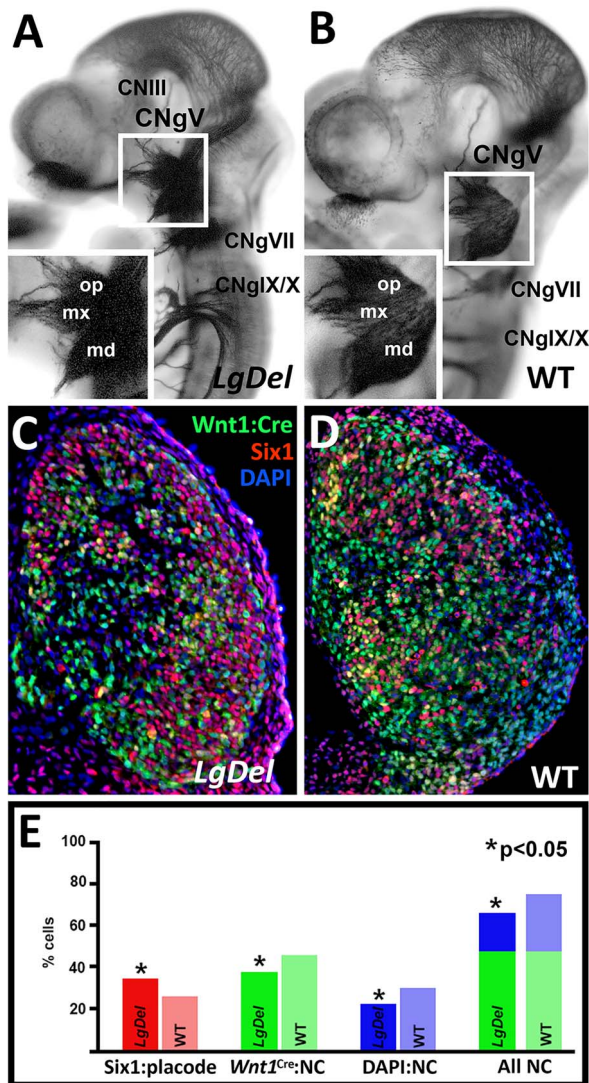


Figure 1. Divergent trigeminal nerve (CN V) differentiation and trigeminal ganglion (CNgV) cellular composition in the *LgDel* mouse model of 22q11.2 deletion syndrome (22q11DS). (A) A E10.5 *LgDel* embryo labeled for the early neuronal and axon marker β III-tubulin, in which CN V axon fascicles extending peripherally from CNgV are sparse and dysmorphic. *Inset*: The differentiation of all three subdivisions of CNgV—op, mx and md—is compromised in concert with altered CN V axon growth. (B) A WT E10.5 embryo, the same gestational age as the *LgDel* in (A), in which there is robust, directed, fasciculated growth of CN V axons from a well-differentiated CNgV. *Inset*: The three subdivisions of CNgV and their associated axons are well defined, compared to the *LgDel* counterpart (A). (C) A transverse section through the *LgDel* E10.5 CNgV labeled immunohistochemically using Six1 (red), which distinguishes cranial placode-associated CNgV cells, Wnt1:Cre recombination lineage tracing (leading to constitutive eGFP reporter expression) for a subpopulation of CNgV neural crest cells (green); DAPI, which we have shown previously (11), identifies a second sub-population of primarily *Foxd3*-expressing neural crest cells (blue). There is a small population of cells identified by both Wnt1:Cre recombination and Six1 immunolabeling. Those cells have been excluded from the quantitative analysis summarized in the histogram in (D). (D) A section through the E10.5 WT CNgV, with cell classes labeled as described for (C). (E) Quantitative assessment of frequency of Six1 in *LgDel* (darker red) and WT (lighter red), Wnt1:Cre associated neural crest (*LgDel*: darker green, WT: lighter green), and DAPI presumed *Foxd3* neural crest cells (*LgDel*: darker blue; WT: lighter blue); asterisks indicate significant differences, $P < 0.05$; ($n = 11$ WT, 9 *LgDel* ganglia from 8 WT, 5 *LgDel* embryos).

approximately 2-fold (i.e. 50%) decreased level (16) in *LgDel* CNgV. In CNgV, we detected 22 of the 28 murine 22q11 orthologues (17) with FPKMs greater than 1 for all but 1, *Rtn4*, a transmembrane protein involved in neurite outgrowth (18). Expression of these 22q11 genes in *LgDel* CNgV decreases by approximately 2-fold as expected (Fig. 2C, solid bars); however, the FDR of only five: *Cldn5*, *Dgcr2*, *Dgcr14*, *Ranbp1* and *Zdhhc8* met the $q < 0.1$ criterion of significance (asterisks, Fig. 2D).

To evaluate whether our microdissected CNgV samples enhance identification of transcriptome differences, we compared our CNgV results with RNA-Seq-measured transcriptomes of whole E10.5 WT and *LgDel* embryos (Fig. 2E). This comparison evaluates selectivity of the microdissection technique for CNgV enriched genes, and potential specificity of the CNgV dataset for detecting transcriptome changes associated with CNgV sensory progenitor, neuronal and glial differentiation rather than those broadly associated with 22q11 deletion at this stage of development. In transcriptomes from two replicates of four whole E10.5 embryos of each genotype, we found a total of 58 differentially expressed genes using the same significance threshold (FDR $q < 0.1$; Fig. 2F and Supplemental Table 3). Within these 58 differentially expressed genes, 17 are orthologues of the 22q11-deleted genes, and all decline in *LgDel* by approximately 2-fold (Fig. 2D, hatched bars). Thus, both datasets—E10.5 CNgV and E10.5 whole embryo—accurately represent the fundamental genotypic distinction: 50% (2-fold) decreased expression of murine 22q11 gene orthologues in *LgDel*. Of the 58 differentially expressed genes in the whole embryo transcriptome comparison, only 5 coincide with those identified as differentially expressed in CNgV transcriptomes (Fig. 2F). Four are 22q11 genes: *Cldn5*, *Dgcr14*, *Ranbp1* and *Zdhhc8*. The fifth is *Hsd3b6*, which is expressed at relatively low levels (mean FPKM, WT: 2.1; *LgDel*:6.3), and apparently upregulated in *LgDel* versus WT CNgV and downregulated in *LgDel* versus WT E10.5 whole embryos. *Hsd3b6* encodes hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 6 known to be localized to adrenal cortical cells (19). The minimal overlap of differentially expressed transcripts in CNgV with those in whole embryos confirms that RNA-Seq data obtained from the microdissected CNgV samples reflect its constituent cell classes and thus describe transcriptional states of cranial sensory neurons in the two genotypes.

Finally, we assessed the functional identities of *LgDel* versus WT CNgV differentially expressed genes. Half of the differentially expressed CNgV genes (84 of 134) could be assigned a known molecular and/or biological function based on Gene Ontology (GO, <http://www.geneontology.org>; Fig. 2G). The differentially expressed genes of known function were significantly over-represented ($P < 0.05$) in a number of broad cell-signaling and metabolic pathways, including several that might contribute to sensory neuron differentiation. These include Slit/Robo signaling (20–22), BMP signaling (23,24), oligodendrocyte myelination (25) and stem cell differentiation (Fig. 2G). Thus, a large but fairly well-defined set of differentially expressed genes, many potentially belonging to functional pathways associated with developmentally regulated signaling and neural differentiation processes, distinguishes the transcriptomes of *LgDel* versus WT CNgV.

Reliable detection of CNgV-associated gene expression

To assess the precision and reliability of our CNgV RNA-Seq data, we confirmed expression of several genes based on limited or enhanced CNgV localization of their encoded protein and then

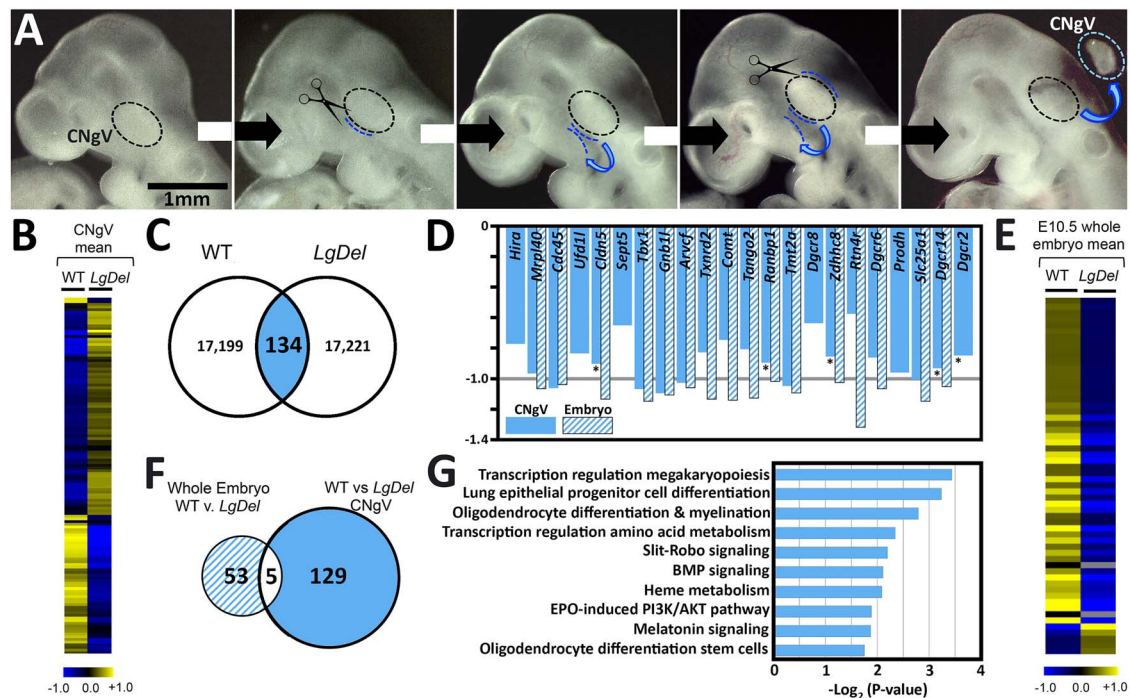


Figure 2. Quantitative characteristics of transcriptome divergence in *LgDel* and WT CNgV. (A) Images of E10.5 mouse embryos showing the step-wise microdissection approach used to harvest WT and *LgDel* CNgV. The dotted oval indicates the location of CNgV, the dotted blue lines indicate the incisions made by fine op dissecting scissors to isolate the ganglion. An isolated ganglion is shown at far right (dotted oval). (B) A heat map showing mean expression differences between WT and *LgDel* CNgV based on RNA-Seq transcriptome analysis of five biological replicates. The scale bar below the heat map (−1.0 to +1.0) is given on a \log_2 scale. (C) Venn diagram showing the distribution of differentially expressed genes in the WT versus *LgDel* CNgV. There are 134 genes with greater expression in either *LgDel* versus WT or WT versus *LgDel*. In addition, there are 9 transcripts in WT and 10 in *LgDel* that are apparently uniquely expressed. Each of these unique transcripts, in either genotype, is expressed at very low frequency and encodes either non-coding RNAs or other non-transcribed mRNAs. (D) Histogram showing detection (non-zero FPKM) and approximately 50% decreased expression levels (2-fold change) of 21 murine orthologues of human chromosome 22q11.2 genes deleted heterozygously in the *LgDel* CNgV (solid bars) and 15 of those orthologues detected non-zero values in the whole E10.5 embryo at a similar level of decreased expression (hatched bars). Of these genes, only five reach our criteria for expression and FDR levels in CNgV (asterisks). (E) A heat map showing average expression differences between whole E10.5 WT and *LgDel* whole embryos. The scale bar below the heat map (−1.0 to +1.0) is given on a \log_2 scale. (F) Venn diagram showing the minimal overlap of differentially expressed genes from the 134 identified in the comparison of WT versus *LgDel* CNgV and the 58 identified in the comparison of WT versus *LgDel* whole E10.5 embryos. There are only five of these genes, and four of them are 22q11 gene orthologues deleted heterozygously in the *LgDel* (see C). (G) A listing of the major GO terms associated with the RNA-Seq datasets for WT and *LgDel* CNgV.

queried the RNA-Seq dataset for mRNA detection of the same genes. *Hox1b* protein is barely detected in CNgV but robustly and selectively localized in the adjacent hindbrain (Fig. 3A, left). There was no obvious difference in pattern or level of *Hox1b* protein detection in WT versus *LgDel* CNgV or hindbrain. The RNA-Seq data paralleled protein expression: *Hox1b* is detected at very low levels in CNgV from both genotypes (FPKM mean 0.37, WT; 0.55 *LgDel*, $q=0.995$; Fig. 3A, right). We then evaluated localization of proteins that are selectively expressed at higher, but varying levels in CNgV at E10.5. *Pax3*, a transcription factor associated with a subset of CNgV neural progenitors, has no discernible difference in protein expression pattern or level in WT and *LgDel* CNgV. Similarly, *Pax3* is detected at equivalent but relatively low levels in WT and *LgDel* CNgV in our RNA-Seq data (WT 22.1; *LgDel* 17.0, $q=0.99$; Fig. 3B). Three additional neural progenitor-associated proteins—nestin, brain lipid-binding protein (*blpb*)/fatty acid-binding protein 7 (*fabp7*) and vimentin—are selectively expressed at low, intermediate and high levels, respectively, in WT and *LgDel* CNgV, based on apparent frequency of labeled cells (Fig. 3C–E, compare right-hand panels in each column). Their mRNAs are detected at similar relative levels with no significant differences between genotypes (*Nes*-94, WT; 74, *LgDel*, $q=0.99$; *Blpb/Fabp7*-218.6, WT; 156.2, *LgDel*, $q=0.69$; *Vim*-748.4, WT; 914.7, *LgDel*, $q=0.99$; Fig. 3C–E, far right). Thus, based

on comparison with an independent detection method for genes differentially expressed in CNgV, our RNA-Seq datasets appear robust and reliable.

Validation of established, cell-class-selective CNgV transcriptional regulators

Four transcription factors are established markers for placode-derived/mechanosensory versus neural crest-derived/nociceptive cell lineages in cranial sensory ganglia including CNgV: *Six1*, *Bmn3a*, *Sox10* and *Foxd3* (11,26–28). Accordingly, each of these transcripts should be detected at substantial levels in both WT and *LgDel* CNgV samples, in register with cell-class-selective protein expression. In addition, these genes may be expressed at quantitatively distinct levels, in register with shifted proportions of placode- versus neural crest-derived cells in *LgDel* CNgV (see Fig. 1). We therefore compared the RNA-Seq values of these four genes with expression values measured by quantitative PCR (qPCR) in a parallel, independent set of 5 E10.5 microdissected, pooled CNgV samples.

We found robust expression levels in the RNA-Seq dataset for the placode-associated gene *Six1*, as well as *Bmn3a*, a marker for CNgV neuronal differentiation (29), whose protein expression

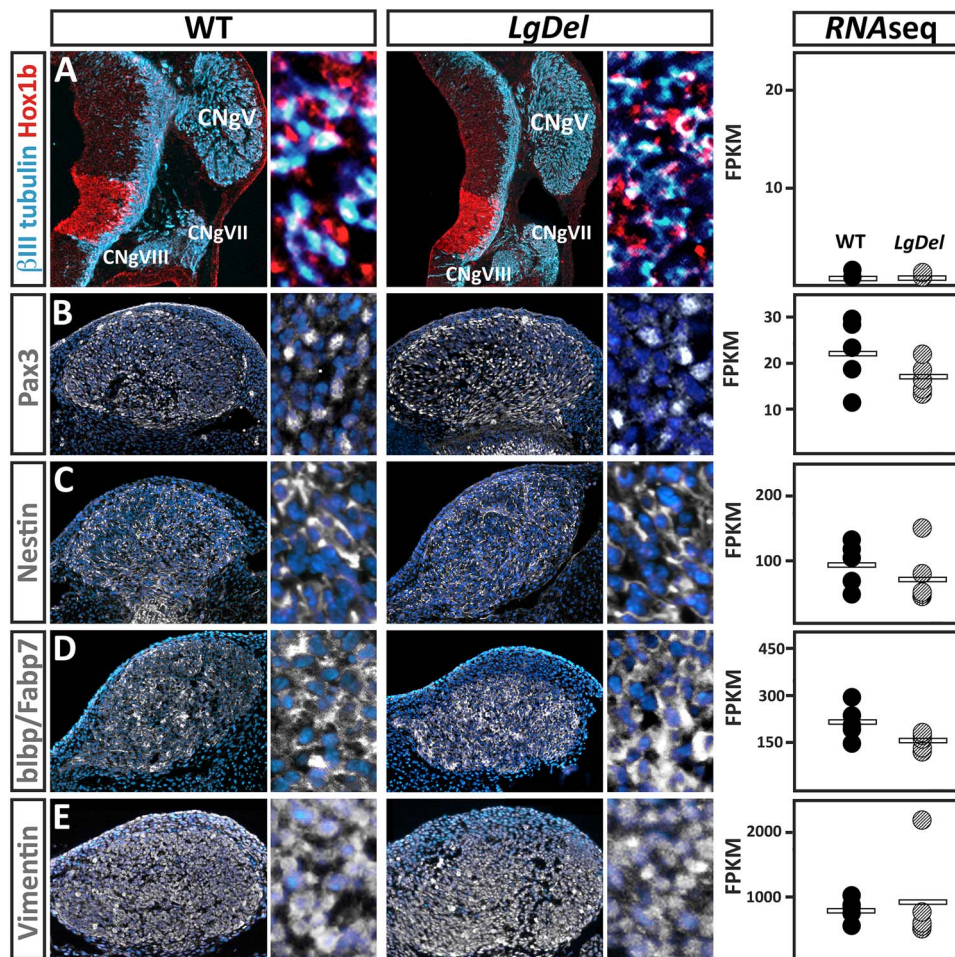


Figure 3. Registration of cellular CNgV protein expression localization at E10.5 and RNA-Seq mRNA detection from micro-dissected CNgV. For the images demonstrating protein expression, the first panel shows the expression pattern of each protein recognized immunocytochemically in CNgV in each genotype, and the adjacent second panel shows the distribution of individual labeled cells for the same protein. (A) Hox1b protein is expressed in a very limited population of CNgV cells but highly expressed in rhombomere 4 (r4) in the hindbrain immediately adjacent to CNgV in both WT and *LgDel*. In parallel, *Hox1b* mRNA is expressed at very low (FPKM < 0.1) but equivalent levels in the WT and *LgDel* CNgV RNA-Seq datasets. (B) Pax3 protein is detected in a subset of CNgV cells in both WT and *LgDel*. Right: Pax3 mRNA is detected at relatively low but statistically indistinguishable levels (FPKM < 5.0) in the WT and *LgDel* CNgV RNA-Seq datasets. (C) Left: Blpb/Fabp1 protein is localized in a somewhat broader subset of CNgV cells in both WT and *LgDel*. Right: Blpb/Fabp1 mRNA is detected at intermediate but statistically indistinguishable levels (FPKM < 100) in the WT and *LgDel* CNgV RNA-Seq datasets. (D) Left: nestin protein is more broadly localized in CNgV cells in both WT and *LgDel*. Right: Nes mRNA is detected at moderate (FPKM < 300) but statistically indistinguishable levels in the WT and *LgDel* CNgV RNA-Seq datasets. (E) Left: vimentin protein is localized to nearly all CNgV cells in both WT and *LgDel*. Right: Vim mRNA is detected at high (FPKM < 1000) but statistically indistinguishable levels in the WT and *LgDel* CNgV RNA-Seq datasets.

coincides with that of *Six1* in the E10.5 CNgV (Fig. 4A and B, top) (11). We also found substantial expression levels in the RNA-Seq data of *Sox10*, whose protein expression nearly completely coincides with *Wnt1:Cre-eGFP* labeled neural crest-derived CNgV cells, and *Foxd3*, an established neural crest marker whose expression identifies *Wnt1:Cre*-negative, *Six1* negative presumed CNgV neural crest cells (Fig. 4A, bottom) (11). *Six1* and *Brn3a* expression levels appear to increase in the RNA-Seq and qPCR data for *LgDel* versus WT CNgV. Increased expression of both genes accords with the increased proportion of *Six1*-labeled cells in *LgDel* CNgV at E10.5 (see Fig. 1) that also express *Brn3a*. Statistical significance, however, was only reached for *Six1* in the RNA-Seq data ($P=0.046$) and for *Brn3a* in the qPCR data ($P=0.03$; Fig. 4B and C, top). In contrast, even though we detected modest differences for the neural crest-associated transcription factors, only that for *Sox10*, measured by qPCR, reaches statistical significance ($P=0.037$). The direction of change of transcript

level, however, an increase, does not accord with the direction of change in neural crest cell frequency, a decrease.

Based on detection of these CNgV lineage/cell-class-selective markers in our RNA-Seq, qPCR and protein localization data, we asked whether the 134 differentially expressed genes detected by RNA-Seq might be potential regulatory targets of these four lineage-associated CNgV transcription factors. We conducted a gene promoter analysis to search for cis-acting binding sites of each transcription factor using the TRANSFAC (30) algorithm with a strict search criterion: 5% maximal matrix dissimilarity within 200 nucleotides upstream sequence from the start of transcription, to identify genes with cognate-binding elements for *Six1*, *Brn3a*, *Sox10* or *FoxD3*. This analysis identified 38 of the 134 (28%) differentially regulated genes as potential *Six1* targets, 0 of the 134 (0%) as potential *Brn3a* targets, 103 of the 134 (77%) as potential *Sox10* targets and 33 of the 134 (25%) as potential *FoxD3* targets (Fig. 4D; Supplemental Table 4). The lack

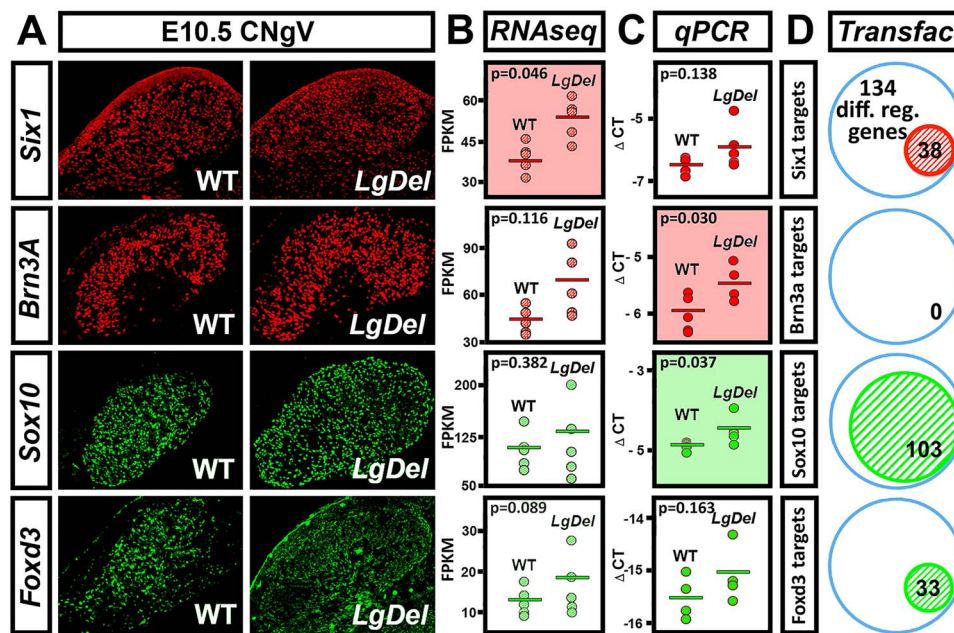


Figure 4. Validation of *LgDel* versus WT CNgV transcriptome comparison based on expression of transcription factors associated with placodal or neural crest-associated CNgV neurons or precursors. (A) Protein expression of Six1, Brn3a, Sox10 and Six1 in the E10.5 CNgV of WT and *LgDel* embryos. (B) RNA-Seq determined expression levels of diagnostic transcription factors associated with E10.5 cranial placode-derived CNgV cells—Six1 and *Brn3a*—and neural crest-derived CNgV cells—Sox10 and *Foxd3*—in *LgDel* and WT CNgV. Expression levels determined by RNA-Seq are presented for each of the five biological replicates of WT and *LgDel* CNgV as FPKM values. (C) Expression levels determined by qPCR in a parallel set of five biological replicates of WT and *LgDel* CNgV pooled samples are presented as delta CT (Δ CT) values. (D) TRANSFAC computational analysis identifies subsets of the 134 genes differentially expressed in WT versus *LgDel* CNgV (see Fig. 2) as potential transcriptional targets for the diagnostic transcription factors associated with either placode-derived or neural crest-derived CNgV cells.

of *Brn3a* targets is perhaps less surprising since it is expressed at the transition from neurogenesis to post-mitotic neuroblast to influence subsequent CNgV neuronal differentiation (31), which has not begun in earnest at E10.5. Together, these data indicate that our RNA-Seq analysis reliably detects established regulators of CNgV identity in both WT and *LgDel*. Moreover, expression changes of some of these genes parallel altered proportions of placode-associated CNgV cells—particularly for placode-associated genes. The relationship of these data to decreased neural crest cell frequency is less clear. Some expression level differences corresponding to quantitative cellular changes may be below the level of reliable detection of these methods. Alternatively, the increased *Sox10* expression in *LgDel* detected by qPCR but not RNA-Seq may reflect a real—yet quantitatively discordant—change in CNgV neural crest transcriptional state due to altered specification, migration, proliferation and cell–cell interactions associated with altered proportions of these cells.

Differentially expressed CNgV genes outside of the 22q11.2 deleted region

Our assessment of the 134 up- or downregulated genes identified several individual candidates that based on presumed functional significance for 22q11 deletion-associated phenotypes, cranial sensory neuron development or known roles in branchial arch and neural crest differentiation were chosen for further validation. We chose genes expressed at fairly high abundance based on FPKM values (>5) in our RNA-Seq dataset, and with substantial magnitude of expression changes (≥ 2 -fold decrease or increase). Based on these criteria, we selected seven additional genes for qPCR analysis in a parallel set of five CNgV pooled samples from each genotype (Fig. 5). Four of these genes appear by RNA-Seq to decrease in expression (Fig. 5A–D):

Josd2, a Josephin-domain containing presumed deubiquitinating enzyme (32); *Lfng*, a Notch signaling intermediate (33); *Atoh1*, a bHLH transcription factor known to regulate sensory and cerebellar neuronal development (34) and *Epha7*, an Ephrin receptor tyrosine kinase with putative functions in neuronal migration, axon growth and guidance (35). However, these four genes all appear to increase (although not statistically significantly) when assayed by qPCR. Two candidates increase significantly (Fig. 5E and F): *Cited4*, a transcriptional co-activator implicated in cardiac myocyte differentiation (36), and *Icam4*, an immunoglobulin/cell adhesion molecule implicated in cytokine signaling and immune cell adhesion (37). Expression levels of these genes also increase in the qPCR dataset, but only *Cited4* reaches statistical significance. Thus, there is both divergence and convergence between expression levels measured in RNA-Seq and qPCR datasets generated from E10.5 microdissected WT and *LgDel* CNgV.

CNgV transcriptome changes reflect altered hindbrain neural crest patterning

Our previous work suggests that patterning of anterior hindbrain rhombomeres (r1/r2), from which most CNgV neural crest progenitors originate, is altered in the *LgDel* embryo. This change, toward a more posterior identity, disrupts several aspects of CNV differentiation (6). To assess whether divergent transcriptional signatures in *LgDel* versus WT CNgV reflect this anterior to posterior (A/P) identity shift (Fig. 6A), we compared our transcriptome results to an existing RNA-Seq dataset (38) that catalogues transcripts associated with cranial neural crest of distinct A/P rhombomeric origins. To maximize compatibility of our data with that from the A/P neural crest transcriptomes, we used EdgeR (39) in addition to CuffDiff analysis to identify differentially

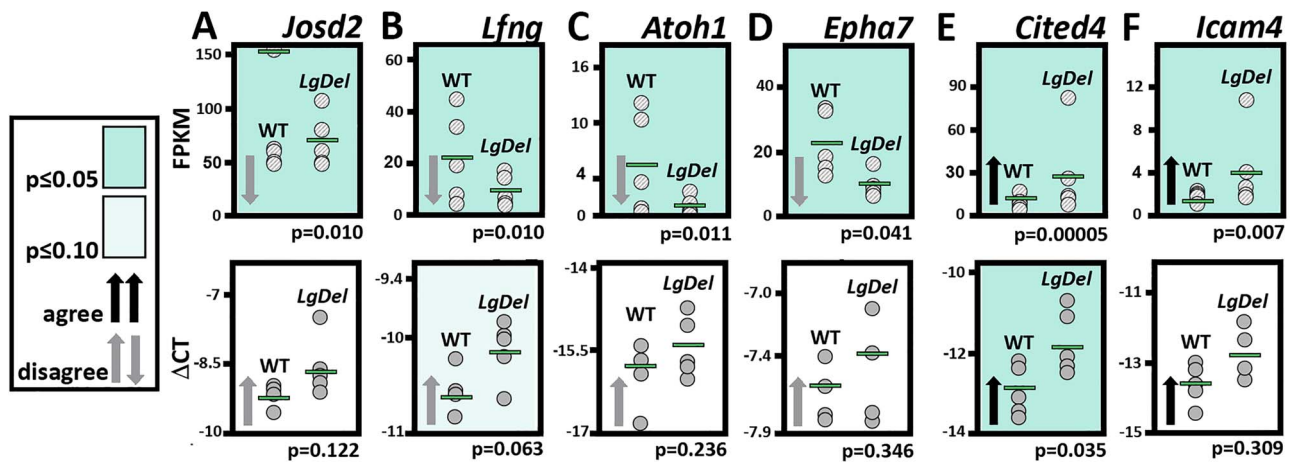


Figure 5. Comparison of expression differences for candidate genes identified by RNA-Seq using qPCR. (A–F) Differential expression validation for six candidate genes, chosen based on potential contributions to CNgV neuronal differentiation from among the full set of differentially expressed genes identified in the RNA-Seq dataset. Individual values (FPKM, top or Δ CT, bottom) are plotted for each gene. Green horizontal bars indicate mean expression values for each gene. Arrows indicate direction of expression change (up- or downregulated), and black versus gray shading indicates whether the qPCR and RNA-Seq mean expression differences are in agree (black) or do not agree (gray). The darker green shading indicates genes for which either the qPCR or RNA-Seq dataset identifies a statistically significant expression difference (P or $FDR < 0.05$). The lighter green shading indicates instances, where an apparent expression difference was detected and had a P value less than 0.1 in the qPCR validation.

expressed genes shared by the neural crest and CNgV datasets. We found 956 differentially expressed transcripts in *LgDel* versus WT CNgV using EdgeR and CuffDiff and confirmed 131 transcripts differentially expressed in the r1/r2 (CNgV-associated) versus r4 (posterior) neural crest.

Thirteen transcripts with significant expression differences, detected by CuffDiff ($P < 0.05$) and/or EdgeR analysis ($P < 0.05$), were common to both datasets. Those from the neural crest dataset whose expression levels are greater in r1/2 than r4 include *Nkx2.9*, *Icam4*, *Ferd3l*, *Trh*, *Shh* and *Sox21*; and those with greater expression levels in r4 versus r1/2 include *Des*, *Acp5*, *Hox1b*, *Cited4*, *Krt14*, *Slc22a4* and *Oc90*. Of these, the expression levels of 12 *LgDel* versus WT CNgV transcripts had a shift consistent with altered A/P identity: five WT r1/r2-enhanced (anterior) transcripts had lower expression in *LgDel* CNgV: *Nkx2.9*, *Ferd3l*, *Trh*, *Shh* and *Sox21* (Fig. 6B, upper left quadrant), whereas all seven WT r4-enhanced transcripts (posterior) had higher expression (Fig. 6B, lower right quadrant). These changes were statistically significant ($P = 0.018$; chi-square). Apparently, there is a shift in A/P gene expression consistent with a shift of A/P identity for the neural crest cells that contribute to CNgV.

Presumably, this distinction is established in hindbrain rhombomeres from which the neural crest directed to CNgV originates and is preserved in neural crest-derived progenitors that constitute a major component of the E10.5 CNgV. Consistent with this interpretation, the A/P shifted gene set includes *Cited4*, the one candidate gene from our RNA-Seq differential expression analysis that was validated as significantly differentially expressed by qPCR analysis (see Fig. 5E). The expression pattern of *Cited4* in the developing head was not defined in the existing literature. Accordingly, we performed *in situ* hybridization (ISH) for *Cited4* on WT and *LgDel* embryos as a further validation. WT ($n = 4$) and *LgDel* ($n = 6$) embryos were incubated in the same vial throughout hybridization, labeling and clearing, and imaged at standard illumination and exposure settings. We found that *Cited4* is selectively expressed in CNgV in both WT and *LgDel* embryos (Fig. 6C). In these embryos, it was difficult to identify consistent expression differences between the two genotypes. Nevertheless, the selective expression of *Cited4* in CNgV, as well

as its association with the shift in A/P rhombomere identities confirms the sensitivity of our RNA-Seq—*Cited4* has not been previously localized to CNgV—and detecting expression changes that accord with developmental disruption in *LgDel* versus WT embryos.

Non-parametric analysis of *LgDel* and WT transcriptome differences

Our analysis of mean expression level differences in E10.5 WT and *LgDel* CNgV replicates indicates changes in several genes that may influence divergent CN V differentiation in the *LgDel*. Nevertheless, validation of many of the differentially expressed genes in parallel samples of CNgV did not consistently confirm predictions based on mean expression comparisons from the RNA-Seq data. Indeed, we noted that a significant number of outliers in sample-by-sample expression levels of many transcripts, especially in the *LgDel* samples, seemed to drive mean expression differences, perhaps erroneously. Statistical assessment of mean expression differences, based on CuffDiff analysis, is known to be impacted by outliers, particularly when they deviate by an order of magnitude or more, as is often the case for *LgDel* values. This raised the question of whether the mean analysis had appropriate sensitivity to identify all significant expression level distinctions between WT and *LgDel* CNgV.

Accordingly, we evaluated our CNgV RNA-Seq dataset using a non-parametric method in which WT and *LgDel* expression values as a group were assigned ranks (1 through 10) based on FPKM detection levels (Fig. 7). Potential median differences between individual expression levels in the two genotypes identified by this analysis were compared using the Mann-Whitney U test (a rank-order test analogous to a two-tailed Student's t test), which compares group differences based on median rather than average values. This approach, which minimizes the statistical effects of outliers, identified approximately eight times the number of significantly changed transcripts (1149) than by the CuffDiff analysis (134; Fig. 7A and Supplemental Table 5), and twice the number of transcripts even when no

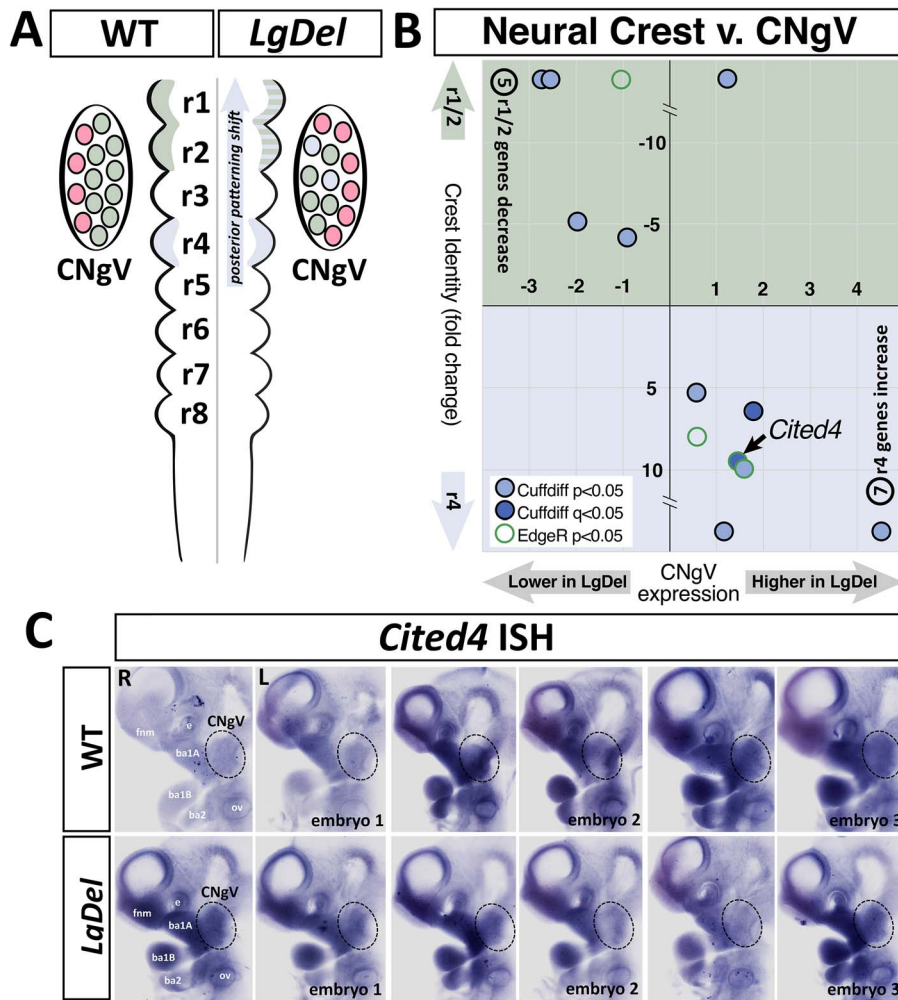


Figure 6. A posterior shift of neural crest-associated genes is detected in the *LgDel* CNgV transcriptome. (A) Schematic of the WT (left) and *LgDel* (right) hindbrain indicating the posterior shift of gene expression in rhombomeres (r)2 and r3 in the *LgDel* as well as the potential for this altered gene expression to be transferred to CNgV due to migration of neural crest progenitors from r2 and r3 into the coalescing CNgV (blue cell icons, far right). (B) A quadrant plot of gene identity (A/P) defined by an independent RNA-Seq analysis (38) and CNgV expression levels detected in our RNA-Seq analysis. Note that *Cited4*, whose expression is apparently increased in *LgDel* CNgV, by both RNA-Seq and qPCR (see Fig. 5), is one of the genes whose A/P expression is shifted. (C) ISH of *Cited4* mRNA in E10.5 WT and *LgDel* embryos. The dotted oval indicates CNgV. These embryos were hybridized together, genotypes distinguished by tail clip.

FDR correction is used (668 transcripts at CuffDiff $P < 0.05$). Nevertheless, there is relatively little overlap between the sets of transcripts identified by the two methods—only 178 of the 668 (26.6%) uncorrected transcripts ($P < 0.05$) and 27 of the 134 (20.1%) FDR corrected transcripts (FDR < 0.10) are also significant by the Mann–Whitney test ($P < 0.05$).

To validate whether this non-parametric approach identifies significantly changed transcripts with at least the same sensitivity as the parametric method, we first assessed the murine orthologues of 22q11 genes that are heterozygously deleted in the *LgDel* mouse. Of the 22 transcripts encompassed by the *LgDel* deletion that are robustly identified in the RNA-Seq dataset (see Fig. 2C), 5 have detectable reduced expression by CuffDiff parametric assessment; however, 20 are identified as significant by the non-parametric analysis (Fig. 7B and Supplemental Table 5). In addition, we assessed expression differences that reach significance or accord with CNgV cellular changes detected by the parametric approach: *Six1* and *Brn3a*. The non-parametric analysis detected differential expression

of the placode-associated *Six1* and *Brn3a* and confirmed that the neural crest-associated genes *Sox10* and *Foxd3* are differentially expressed (Fig. 7C). Next, we asked whether a subset of transcripts identified by the non-parametric approach might be robustly replicated by qPCR from additional pooled samples. We assessed 10 differentially expressed genes with varying levels of significance based on our non-parametric analysis: five upregulated (Fig. 7D) and five downregulated (Fig. 7E); only 4/10 of these additional transcripts were significant by both parametric and non-parametric analyses of the RNA-Seq dataset. Our parallel qPCR analysis of these transcripts showed that the non-parametric approach is not more reliable; only one of the transcripts (*Mt2*; Fig. 7, shaded histogram) was significantly different (*LgDel* $>$ WT; $P = 0.047$) in the independent qPCR analysis and the non-parametric analysis (Supplemental Table 5). Apparently, non-parametric statistics may be a useful adjunct for identifying differentially expressed genes, based on robust identification of *LgDel* deleted transcripts. Nevertheless, it appears that a high level of variability is a key feature of the

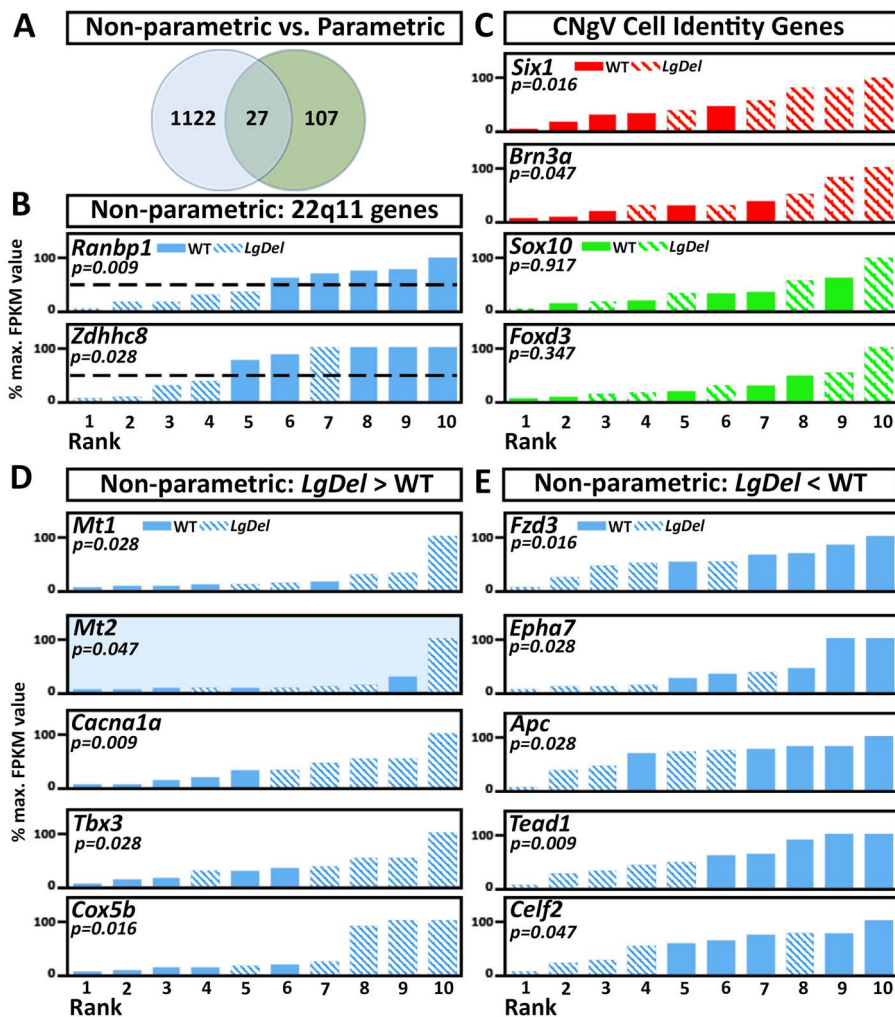


Figure 7. Non-parametric analysis of WT versus *LgDel* CNgV transcriptomes detects a greater number of significantly expression level differences in the two genotypes. (A) A summary of the number of significantly differentially expressed genes detected using non-parametric, rank-order based statistics and the Mann-Whitney *U* test of significance in the WT versus *LgDel* CNgV transcriptome. Nearly 10 times as many genes—1149—are detected at significance levels of $P < 0.05$ or less as compared with 134 detected using the previously described CuffDiff analysis, but only 27 transcripts are identified by both methods. (B) Non-parametric rank-order analysis detects significant 50% decrements in 22q11 gene expression with greater sensitivity. The most (*Ranbp1*) and least (*Zdhhc8*) abundant significantly different 22q11 transcripts are shown here. These values and all others in this figure are plotted in rank order, 1 through 10, left to right, and the expression values across both genotypes are displayed as percentage of the maximum expression value (100%). The dotted line indicates the 50% expression level. Mann-Whitney significance (*P* value) is given in italics. (C) Non-parametric analysis detects changes in expression levels of four diagnostic placode versus neural crest associated genes with accuracy similar to the parametric analysis. (D) Novel genes whose expression is detected as significantly increased based on non-parametric analysis in our RNA-Seq dataset. Mann-Whitney *P* value is given in italics. The shading (*Mt2* non-parametric analysis histogram) indicates that qPCR assessment validated the RNA-Seq detected expression difference ($P = 0.047$; $n = 5$ pooled CNgV replicates/genotype). (E) Novel genes whose expression is detected as significantly decreased based on non-parametric analysis in our RNA-Seq dataset, presented as above. qPCR validation of transcripts shown in (D) and (E) is shown in [Supplementary Table 5](#).

LgDel transcriptome, and not merely measurement ‘noise’ that can be filtered out.

Significantly increased transcriptome variability in *LgDel* CNgV

The consensus of multiple clinical studies of 22q11DS patients (40–43) as well as analyses in mouse models (17,44), including our previous assessment of CNV anomalies that prefigure disrupted S/F/S in *LgDel* mouse pups (6), is that phenotypic variability is a hallmark of 22q11 deletion. Thus, it seemed possible that difficulties in confirming some—but not all—of apparent expression differences from the RNA-Seq dataset in independent samples with alternative methods might reflect a fundamental distinction between the transcriptomes of E10.5 CNgV cells

from WT versus *LgDel*: there may be biologically established statistically verifiable greater variation in expression levels throughout the entire set of expressed genes in *LgDel* due to the apparent 50% change in 22q11 gene dosage. If this is the case, quantitative evidence of this variability should remain despite microdissection intended to limit cell classes being profiled and pooling intended to diminish or eliminate non-biological variability in expression levels.

We therefore compared variability of expression levels on a gene-by-gene basis in the five WT CNgV biological replicates with that in the five *LgDel* CNgV replicates (Fig. 8A). Inspection of the heat maps of each of the individual WT versus *LgDel* samples suggests that there may be greater variation of expression for each gene across the *LgDel* CNgV transcriptome. There seems to be far more frequent differences in magnitude

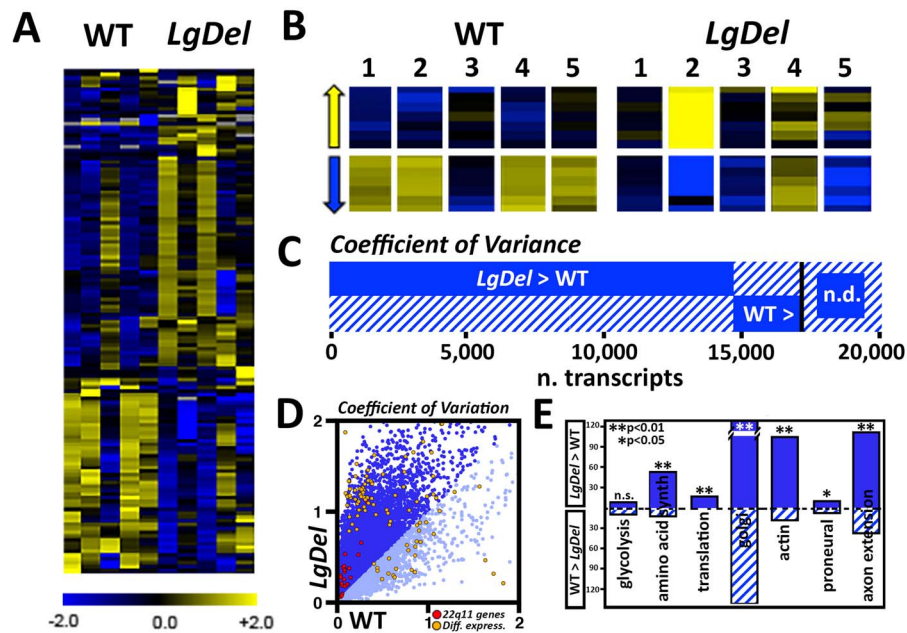


Figure 8. Transcriptome variation as a quantitative phenotype distinguishes *LgDel* and WT CNgV. (A) Heat maps for each of the five biological replicates of WT and *LgDel* CNgV assessed by RNA-Seq. Although there is some variation in the 5 WT replicates (e.g. compare replicate 3 with other WT samples), the variation in the 5 *LgDel* samples appears greater. The scale bar below the heat map is given on a \log_2 scale. (B) Higher resolution view of blocks from the heat map for both up- and downregulated genes confirms the impression of greater variability in the *LgDel* samples. (C) Summary plot of the proportion of 17 128 total genes with non-zero reads with a higher coefficient of variance (CV) in *LgDel* (14 996) than WT (2187), as detailed in Supplemental Table 2. (D) Scatterplot comparing CV in WT (x-axis) versus CV in *LgDel* (y-axis). Darker blue indicates higher CV in *LgDel* versus lighter blue for transcripts with higher CV in WT. 22q11 orthologues all have higher CV in *LgDel* and are shown in red; the subset of 72/134 significantly differentially expressed genes with higher CV in *LgDel* is shown in green. (E) Graphic representation of increased stochastic variation in *LgDel* versus WT (asterisks indicating significant differences, chi-square) among most, but not all of a subset of GO categories. These categories include gene sets associated with fundamental cellular (e.g. glycolysis, mitochondria) and neuronal differentiation mechanisms (e.g. proneural bHLH genes).

of expression differences, for both over-expressed (Fig. 8B, top) and under-expressed genes (Fig. 8B, bottom), as well as those that are expressed at equivalent values. To verify this impression quantitatively across the entire 17 000-plus gene set, we did a 2×2 chi-square analysis for CNgV genes whose mean expression differed significantly in WT versus *LgDel*, as well as for genes whose mean expression did not differ between the two genotypes. We found that there is far greater variability in expression levels across all CNgV expressed genes in *LgDel*. Of the 17 190 expressed genes common to both genotypes, 14 996 have significantly greater variable expression levels in *LgDel* CNgV versus 2187 in WT with only 7 genes varying equally (Fig. 8C and D), based on the coefficient of variation calculated across the 5 biological replicates for each genotype. This difference in the number and level of variable expression between *LgDel* and WT CNgV is significant ($P < 0.00001$; chi-square).

Greater variability extends across most classes of genes, including 22q11 orthologues: 20 of these genes had a higher coefficient of variation in *LgDel* CNgV (Fig. 8D, red dots), and this difference between *LgDel* and WT was also significant ($P < 0.000007$, chi-square). Similarly, the 134 genes found to be differentially expressed in *LgDel* versus WT CNgV (Fig. 8D, orange dots; $P < 0.00009$). In addition, we queried several additional GO categories and found that with the exception of genes associated with glycolysis, stochastic variation was greater in *LgDel* CNgV for several fundamental cellular pathways: amino acid synthesis, protein translation, Golgi apparatus structure and function, actin dynamics, as well as two additional neural development mechanisms: bHLH proneural genes expressed during neural differentiation and axon extension genes (Fig. 8E). Apparently, heterozygous deletion of mouse 22q11 gene orthologues

results in a broad, significant increase in statistically verifiable transcriptome-wide transcriptional variability, extending to many—but not all—functional gene subsets expressed in CNgV. This suggests that an essential dimension of the distinctions between WT and *LgDel* CNgV transcriptomes, in addition to expression differences of particular genes, is increased stochastic variation of overall transcriptional state. Our RNA pooling strategy, which is thought to reduce biologic variability and increase detection power for differentially expressed genes (12,45), likely underestimates stochastic variation observed in *LgDel*. CNgV transcriptional instability, perhaps due to a cell-by-cell basis, may contribute to phenotypic change and may also indicate a potential general mechanism for aspects of phenotypic variability seen in 22q11DS mouse models (16) as well as individuals with 22q11DS (41).

Discussion

The transcriptomes of differentiating CNgV progenitors and sensory neurons are quantitatively distinct in E10.5 *LgDel* versus WT embryos, shortly after the ganglion has coalesced as a distinct structure, in parallel with altered CN V differentiation and CNgV cellular composition. Our transcriptome comparison provides enhanced tissue specificity and statistical resolution of changes that ultimately must reflect the 50% diminished expression level of 22q11 genes in CNgV and related structures that contribute cells to the ganglion: the cranial placodes and hindbrain. Additional quantitative analysis of candidate genes whose expression differs in *LgDel* versus WT CNgV validates some but not all of the RNA-Seq comparisons. Among the validated genes, changes in *Six1* and *Cited4* expression levels accord with cell biological

changes in *LgDel* CNgV or in the hindbrain from where CNgV neural crest progenitors originate. In addition, we identified statistically robust increased variability in expression levels of the majority of the over 17 000 genes expressed in common in *LgDel* and WT CNgV as an essential divergent feature of the *LgDel* versus WT CNgV transcriptomes. We suggest that 22q11 deletion-related CN V differentiation phenotypes in *LgDel* originate in part from this increased stochastic variation across much of the *LgDel* CNgV transcriptome. Such divergence may destabilize programs for CNgV progenitor division or sensory neuron differentiation and disrupt initial oropharyngeal sensory-motor circuit development, including that essential for S/F/S, in 22q11.2DS.

Comparing embryonic CNgV transcriptomes

Our micro-dissection approach for isolating embryonic CNgV mRNA is novel. Several observations reinforce the robustness of the data generated using this approach. First, the number of expressed genes detected consistently in five biological replicates from each genotype is substantial and approximately equivalent: 17 221 in *LgDel* and 17 199 in WT. Apparently, CNgV cells in both genotypes are viable and actively transcribing a large number of genes. Second, we confirmed our previous validation of microdissected *LgDel* and WT CNgV (6) based on parallel detection of selective protein expression in E10.5 CNgV sections and mRNA in our RNA-Seq data. Third, the approximate 50% expression decrement of deleted 22q11 genes in *LgDel* CNgV versus the same genes in WT adds confidence. Fourth, quantitative cell biological changes in proportions of placode-versus neural crest-derived CNgV cells in the two genotypes are paralleled by expression level changes of relevant cell-class associated genes. Finally, an independent RNA-Seq dataset of a related subpopulation of cranial neural crest cells—derived from r1/2, which generates most of the neural crest component of CNgV—detects expression changes of several genes in our CNgV RNA-Seq dataset. Thus, our approach has precision, specificity and reliability to generate robust data to test specific hypotheses of transcriptional distinctions underlying early divergent CNgV differentiation in *LgDel* versus WT embryos.

Transcriptome divergence, candidate genes and independent validation

Our RNA-Seq analysis distinguishes the transcriptomes of early CNgV sensory neurons or their precursors in *LgDel* versus embryos at a critical stage of early CN V differentiation. The number of non-zero reads shared by these samples, 17 190, suggests that 68% of the approximately 25 059 mouse genes with protein sequence data (46) are expressed at some level in CNgV by E10.5. Our microdissection-based approach enriches for genes that are selectively expressed in CNgV, and indeed, we identify several significantly enriched pathways associated with trigeminal sensory neuron differentiation. *Slit/Robo* (20–22) and BMP signaling (23,24) are both established regulators of neural crest progenitors as well as trigeminal axon growth and branching. In addition, the enrichment of pathways associated with oligodendrocyte progenitor proliferation and differentiation may reflect commonalities of cranial neural crest cells with Schwann cell precursors, which are also of the neural crest lineage and generate peripheral glial cells among their many progeny (24). Thus, the informatics comparison of *LgDel* and WT CNgV transcriptomes yields an informative

framework for assessing molecular foundations of trigeminal sensory neuron development and its divergence in *LgDel* embryos.

We tested several hypotheses generated by our biological observations of *LgDel* CN V developmental divergence. First, we found that differentially expressed genes include transcriptional regulators of CNgV cranial placode—*Six1* and *Bm3a*—as well as neural crest—*Sox10* and *Foxd3*—derived populations. These differences accord with a developmental/cellular distinction between WT and *LgDel* CNgV: a proportionate increase in *Six1* mRNA and *Six1*-expressing placode-associated cells in *LgDel*. Moreover, analysis of regulatory sequences of the differentially expressed genes shows that significant subsets of these 134 genes are potential targets of three of the four transcriptional regulators that are ‘cardinal’ markers for placode and neural crest components of CNgV: *Six1*, *Sox10* and *Foxd3* (11,26–28).

Our evaluation of additional hypotheses yielded mixed results. We assessed RNA-Seq-identified candidate genes by qPCR, based on potential roles in progenitor proliferation, neuronal differentiation, axon growth and guidance. For each gene we analyzed, qPCR analysis detected expression at roughly comparable levels in parallel pooled samples collected and processed identically to those used to generate the RNA-Seq datasets. Thus, we found no false positive signals in our RNA-Seq data. Nevertheless, increased expression of only one these genes, *Cited4*, was confirmed at $P < 0.05$ by both RNA-Seq and qPCR. In addition, *Cited4* was further validated as a novel CNgV-expressed gene by ISH in both WT and *LgDel* embryos. Thus, although our RNA-Seq comparison, with multiple biological replicates, provides a high resolution ‘screen’ for total transcriptome differences that underlie *LgDel* versus WT CNgV developmental divergence, it does not do so with absolute resolution on a gene-by-gene basis. Nevertheless, as demonstrated by our data on *Cited4*, RNA-Seq comparisons can be used to identify novel CNgV-expressed genes and generate testable hypotheses of altered expression of relevant candidates in *LgDel* versus WT. Such transcriptome-based hypotheses can be tested further by rigorous complimentary experiments.

Despite some limitations, our RNA-Seq data confirmed differential regulation of two genes that identify CNgV just after the ganglion has coalesced from placode and neural crest-derived cell populations. *Six1* and *Cited4* may contribute to distinctions in *LgDel* versus WT CNgV lineage and subsequent sensory neuron differentiation. *Six1* is a key transcriptional regulator of cranial placode specification and differentiation of placode-derived cranial mechanosensory neurons. Our RNA-Seq analysis detected a significant increase in *Six1* mRNA, parallel to a significant proportionate increase in *Six1*-expressing *LgDel* CNgV cells. This agreement between transcriptome and cell biological change is further reinforced by the concentration of *Six1* transcriptional targets among the 134 genes that are significantly differentially expressed in *LgDel* versus WT CNgV. This apparently enhanced regulation may reflect the increased number of placode-derived presumed CNgV cells, or altered differentiation due to increased *Six1* expression in *LgDel*. Less is known about primary developmental functions of *Cited4*, a CBP/P300 interacting transcriptional cofactor expressed in multiple embryonic and adult tissues including heart and mammary glands (47). Complete loss of *Cited4* function is embryonic lethal; nevertheless, there is evidence of disrupted early skin differentiation (48). In addition, *Cited4* inactivation is associated with oligodendroglial tumorigenesis (49). Thus, increased *Cited4* levels as well as anomalous A-P

expression in *LgDel* (6) might modify proliferation, neurogenesis or gliogenesis in *LgDel* CNgV neural crest-derived progenitors, especially since our GO analysis identifies several pathways related to gliogenesis as highly enriched in the E10.5 WT and *LgDel* CNgV transcriptomes. Thus, the two validated expression changes in our RNA-Seq analysis suggest potential downstream consequences for the differentiation of two key classes of CNgV cranial sensory neurons: placode-derived mechanosensors and neural crest-derived nociceptors.

Stochastic transcriptional variation is a key 22q11 deletion phenotype

The most robust distinction between the *LgDel* and WT CNgV transcriptomes is greater variation of *LgDel* gene expression levels. These results parallel one other transcriptome analysis that compared 22q11DS model mice (*Df(16)A^{+/-}*: a deletion that parallels *LgDel*) with WT mice (50). In this microarray analysis, only one candidate with a modest transcriptional change was identified and validated in the developing cerebral cortex in the context of substantial additional variability. Similarly, an analysis of 22q11-deleted humans carrying either the 1.5 MB minimal critical or typical 3 MB deletion identified variability as an essential transcriptome characteristic in 22q11 deleted individuals (51). This common feature is unlikely to reflect ‘noise’ in the datasets. Instead, we suggest that transcriptome-wide stochastic expression variability due to 22q11 deletion results in cell-by-cell and embryo-by-embryo differences in development and function. In embryos, these differences amplified by local distinctions in cell signaling states and divergent gestational experience (maternal diet, stress, environmental exposures) destabilize optimal developmental programs for *LgDel* CNgV cell fates.

The quantitative expression variability we detect in CNgV may be similar to ‘fate drift’ described recently based on stochastic expression level variation identified by single-cell transcriptome analysis for pancreatic cell classes as a function of aging (52). In this instance, an ‘environmental’ factor—aging—apparently introduces instability in regulation of expression levels of multiple genes for which there are presumably two genomic copies. Copy number variation may increase stochastic variability. The kinetics of expression initiation and termination, which are thought to be buffered by normal copy numbers, may enhance expression level variation when copy number is greater or less than 2, potentially on a cell-by-cell basis (53). In the context of hChr.21 duplication, combined effects of allelic selection and variable transcription kinetics lead to cell-by-cell differences in expression levels of duplicated Chr.21 genes as well as diploid loci (54). The variation we report here may reflect similar mosaic expression regulation of heterozygously deleted 22q11 genes as well as other genes—individual cells may be effectively null for some loci, haploinsufficient or diploid for others. Such mosaicism could lead to variable, divergent differentiation across the entire population of early differentiating CNgV cells and provide an initial pathogenic departure for anomalous circuit development underlying perinatal dysphagia and other oropharyngeal dysfunction in 22q11DS.

The increased frequency of stochastic transcription variation we identify in *LgDel* CNgV is substantial; however, differences in expression levels of individual genes, though statistically significant, are far more modest and in some cases difficult to validate using additional methods. Quantitative measures of

expression in tissue samples, including those we identify in carefully microdissected CNgV, may be complicated by such instability of individual cell-by-cell expression levels. Thus, there may be even greater distinctions in transcriptional state from cell to cell in *LgDel* CNgV. Nevertheless, across the entire relatively small population of CNgV cells at E10.5, there are quantitatively verifiable differences in gene expression as well as statistically significantly increased overall stochastic variability in *LgDel*, presumably reflecting the consequences of 50% diminished expression of heterozygously deleted 22q11 gene orthologues. Little is known of the deleterious—or perhaps beneficial—effects of locally variable gene expression in individual cells for any cell class in the developing or mature nervous system. Our findings highlight the need for further assessment of this quantitative feature of transcriptional states in genetically typical as well as mutant individuals during development and in the adult. Future analysis of single-cell sequencing data combined with highly sensitive, quantifiable expression localization in cells within their native tissues may further delineate the nature, mechanisms and potential developmental or functional significance of normal stochastic gene expression as well as that due to 22q11 deletion as well as additional single gene or gene dosage disorders.

Materials and Methods

Embryo microdissection, sample collection and RNA extraction

C57Bl/6N WT dams, obtained directly from the animal vendor (Charles River), carried timed pregnancies sired by *LgDel* males, also C57Bl/6N. The *LgDel* males from our colony descend from a lineage of over 20 generations. Each generation of *LgDel* studs inherits the deletion paternally, via matings with C57Bl/6N WT dams from the vendor to avoid inbreeding. For this study, pregnant dams were sacrificed by rapid cervical dislocation on gestational (Embryonic) day E10.5 (vaginal plug = E0.5) and embryos quickly collected in RNase-free HEPES buffer. In C57Bl/6 mice, the average gestational time in days is 19.26 ± 0.04 days (55). Thus, the stage at which we collect embryos can be considered late mid-gestation if one defines the mid-point gestational day as E9.6, and the mid-gestation period as ± 1.0 days around this mid-point. CNgV was harvested by microdissection from these E10.5 WT and *LgDel* embryos. The two ganglia from each embryo were placed into RNAlater (Ambion) and stored separately until genotyping from additional tissue from that embryo was complete.

Once genotypes were established, pooled CNgV samples were generated to yield five biological replicates for each genotype (WT, *LgDel*). One biological replicate consisted of a pool of at least six ganglia from at least three embryos of the same genotype over several litters. The numbers of cells in mouse CNgV at E10.5–E11.0, the earliest age when such estimates have been reported in the literature, is between 5000 and 6000 (56,57), a number confirmed by our own quantitative estimates. We see no size difference or obvious cell loss in *LgDel* CNgV at E10.5 (see Fig. 1). Thus, 30 000–36 000 cells, at most, comprise each pooled sample for each genotype. Accordingly, we used all of the RNAs isolated from each pool for subsequent transcriptome sequencing. Total RNA from each pool was extracted using the single-step acid guanidinium thiocyanate-phenol-chloroform method (10) and subjected to RNA quality control (QC) with RIN scores ≥ 8 (Agilent 2100 Bioanalyzer, Agilent Tech., Santa Clara, CA, USA) using standard protocols (6). Similar methods were used for whole

embryo samples (two pools of WT and *LgDel*, consisting of four embryos each).

Embryo immunocytochemical analysis

A separate set of E10.5 WT and *LgDel* embryos was collected to assess cranial ganglion and nerve developmental phenotypes and cell-class-selective protein expression within CNgV. One set of embryos was genotyped and then labeled whole for the early neuronal marker β III-tubulin (mouse anti- β III tubulin, Covance), which identifies both peripheral sensory ganglion neurons and their growing axons. A second set of *LgDel* and WT embryos also carried *Wnt1:Cre* recombinase and eGFP nuclear reporter alleles heterozygously (10). These embryos were processed for cryomicrotomy and immunolabeled for Six1, a marker of placode-derived cranial sensory neurons (rabbit anti-Six1, Proteintech) (11) and an antibody against eGFP (chicken anti-GFP, Abcam) to amplify the reporter signal established by *Wnt1*-mediated recombination in CNgV cells presumed to originate from the neural crest. Sections were imaged with a Leica tiling video microscope. Six1 (placode-derived) and *Wnt1:Cre* (neural crest derived) labeled cells were counted blind to genotype on digitized micrographic images to estimate relative frequencies (11). Primary antibodies directed against Brn3a (mouse, Millipore), Sox10 (goat, Santa Cruz) and Foxd3 (mouse, Thermo Scientific) as well as Hox1b (rabbit, Covance), Pax3, blpb/fabp7 (both mouse monoclonal antibodies, Developmental Studies Hybridoma Bank), nestin (mouse, BD Sciences) and vimentin (rabbit, Enzo Life Sciences) were used to label E10.5 sections of CNgV using the same methods. Primary antibody labeling was visualized using Alexa Fluor 488-, 546- and 647-conjugated secondary antibodies (Molecular Probes).

Library construction and sequencing

Paired-end libraries were constructed according to the Illumina protocol for the HiSeq2000 platform. Each pooled CNgV RNA sample, defined as a single biological replicate, was fragmented prior to cDNA conversion to ensure transcript coverage. WT ($n=5$ replicates) and *LgDel* ($n=5$ replicates) were subjected to sequencing (~100 million paired-end reads/replicate, 100 bp read length) on the Illumina HiSeq2000 platform. For WT RNA-Seq, 111–123 million paired-end reads were generated after QC filtering, and 97.3–97.9% of the reads aligned to the mouse genome reference sequence. For *LgDel* RNA-Seq, 77–137 million paired-end reads were generated after QC filtering, and 94.3–97.8% of the reads aligned to the mouse genome reference sequence.

Data processing

Raw sequence data were processed using Illumina's RTA and CASAVA pipeline software, which include image analysis, base calling, sequence quality scoring and QC.

Sequencing datasets were processed through an in-house transcriptome analysis pipeline, employing HISAT/Cufflinks and SAMtools packages, including recent sub-developments (58–62), as described previously (63,64). The quality-filtered sequencing reads are aligned against the latest version of the mouse reference genome (GRCm38/mm10) using HISAT2 (v.2.1.0). These alignments were indexed using SAMtools (v.1.4) and aligned reads assembled into transcripts using Cufflinks (v.2.2.1) (58). The minimum possible spliced transcripts that explain all

aligned read pairs were assembled; abundance was calculated as fragments per kilobase of exon per million fragments mapped (FPKM). Differential expression analysis was performed using two strategies in parallel: the CuffDiff utility of Cufflinks (58) and the outlier-resistant EdgeR-robust package (39,65). The expression profiles and the final transcript predictions of interest were examined individually using Integrated Genome Viewer (v.2.5) (66).

Functional and enrichment analyses

GO categories, pathway enrichment and network analysis were assessed using MetaCore (Clarivate Analytics), Ingenuity Pathway Analysis (Qiagen Bioinformatics) and the AmiGO 2 platform (<http://amigo.geneontology.org/amigo>).

Statistics

Quantitative estimation of the expression levels of the different isoforms between and within the genotype groups was performed using the Cufflinks package, CuffDiff, which contains normalization modules supporting across-sample comparisons. In addition, ANOVA was applied to define significantly differentially regulated transcripts, and *P* values were corrected for multiple testing using False Discovery Rate (FDR) (67). Unless otherwise noted, FDR of 10% ($q < 0.1$) was considered significant for all analyses.

qPCR and ISH validation

We validated the quantitative expression changes identified by RNA-Seq using qPCR and ISH for mRNAs significantly up- or downregulated in *LgDel* versus WT CNgV. qPCR primers were designed for all of the genes (Supplemental Table 1). Parallel sets of 5 WT and 5 *LgDel* microdissected, pooled CNgV samples were prepared for qPCR analysis. For ISH, digoxigenin-labeled probes were synthesized using plasmids containing the full-length *Cited4* open-reading frame sequences (OriGene). Whole-mount ISH was performed using E10.5 WT and *LgDel* embryos fixed in 4% paraformaldehyde overnight and stored in 100% methanol. Embryos were re-hydrated into PBS + Tween 20 buffer before probe hybridization overnight at 65°C. After several washes, embryos were incubated in 10% sheep serum in TBS + Tween 20 (TBS-T) buffer followed by overnight incubation at 4°C with anti-digoxigenin-AP antibody (Roche) in blocking solution to detect bound probe. Embryos were washed several times with TBS-T buffer and developed in BM-Purple (Roche). Embryos were photographed using a Leica M420 stereoscope fitted with a Leica DFC480 camera. ISH experiments were performed on a minimum of five control and five mutant embryos.

Supplementary Material

Supplementary Material is available at HMG online.

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