DIGEORGE SYNDROME PHENOTYPES REFLECT DISRUPTED INTERACTION BETWEEN INDUCTIVE SIGNALS AND 22Q11 GENES

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ABSTRACT

DEEPAK GOPALAKRISHNA: DiGeorge Syndrome phenotypes reflect disrupted interaction between inductive signals and 22q11 genes
(Under the direction of Anthony LaMantia)

We asked whether similar phenotypes that result from diminished 22q11 gene dosage and altered Sonic Hedgehog (Shh), Fibroblast Growth Factor (Fgf), Retinoic Acid (RA) or Bone morphogenetic protein (Bmp) signaling reflect interactions between 22q11 genes and these cardinal morphogenetic signals. When Shh, RA, Fgf, or Bmp signaling is disrupted, expression levels, but not patterns, of several 22q11 genes change in mid-gestation mouse embryos, with most substantial changes associated with altered Shh signaling. When 22q11 gene expression is diminished in mouse embryos by a deletion similar to that in DiGeorge/22q11 Deletion Syndrome (22q11DS), expression of a subset of Shh-, RA-, and Bmp-, but not Fgf-related signaling molecules is altered, with several RA intermediates most substantially changed. Shh and RA signaling, quantified using reporter mice, is altered in the brain or heart of 22q11 deleted, but not *Tbx1*$^{+/-}$embryos, even though diminished *Tbx1* dosage has been suggested as essential for 22q11DS phenotypes. Brief pharmacological disruption of Shh signaling in mid-gestation 22q11-deleted or wild type, embryos leads to severe dysmorphology. Disrupted RA signaling introduces or enhances brain and heart phenotypes in 22q11-deleted but not wild type or *Tbx1*$^{+/-}$ embryos. Thus, early

heart and brain morphogenesis depends on interactions between Shh and RA signaling and 22q11 gene dosage. Apparently, 22q11 gene dosage sustains normal morphogenesis by maintaining a dynamic range of signaling that, when altered, may intensify cardiovascular and CNS phenotypes in 22q11DS.

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INTRODUCTION - I

Patients with a heterozygous deletion of 30-40 genes on chromosome 22 (22q11 deletion syndrome) exhibit significant variability of cardiovascular and brain phenotypes. This dissertation will focus on the analysis of the genetic, cell biological and molecular mechanisms of variability in the abnormal heart and brain development associated with 22q11 deletion syndrome.

22q11Deletion Syndrome: consistent genetic cause and variable phenotypes

22q11 deletion syndrome [DiGeorge syndrome (1)/ Velo-cardio-facial syndrome (2): 22q11DS] is the consequence of a hemizygous microdeletion on the proximal portion of the long arm of human chromosome 22. 22q11DS is the most common (1:2000) multiple anomaly syndrome resulting from copy number variation in humans (3, 4). The resulting wide spectrum of anomalies is due to a change in dosage of multiple syntenic genes as opposed to rare recessive single gene mutations or larger scale chromosomal rearrangements. This makes 22q11DS an ideal model system to study the role of gene networks and gene dosage in early morphogenesis of various embryonic structures. Approximately ~90% of 22q11DS patients have a similar 3 Mb (million base pair) deletion within the 22q11.2 region (5, 6) which contains about 40 genes (between LCR-A and LCR-D; Figure 1). A smaller percentage (8%) of patients carry a nested 1.5 Mb

deletion, spanning 30 genes (LCR-1 to LCR-B; Figure 1). Loss of the critical (1.5Mb) region seems to be sufficient to recapitulate penetrance and severity of the phenotypes resulting from the loss of the larger 3Mb region. Similar phenotypes resulting from non-overlapping deletions within the 22q11 region indicate that no single gene in the region is fully responsible (7, 8). In addition, there have been no single heterozygous gene mutations within the 22q11 region that have a similar phenotypic profile to the full deletion (Figure 1 – Indiv K/O). Thus, studying a contiguous gene syndrome like 22q11DS, whose genomic lesion and range of developmental phenotypes can be modeled in mice, will allow mechanistic molecular insight into multigenic networks of gene interactions that are disrupted in a variety of congenital developmental defects. I have used mouse models of 22q11DS to elucidate the interactions between the multigenic genomic lesion and M/E signaling that are disrupted in 22q11DS resulting in altered tissue morphogenesis.

22q11DS is characterized by a spectrum of phenotypes in multiple organ systems whose early embryonic development is driven by mesenchymal-epithelial interactions (M/E interactions). Common—and clinically significant—manifestations of the disorder include craniofacial (cleft palate, velo-pharyngeal insufficiency), cardiovascular anomalies, immunodeficiency (thymic aplasia), short stature and hypocalcaemia (4, 9, 10). The syndrome has also drawn considerable attention because of its association with number of cognitive and behavioral impairments including attention deficit disorder, autism, schizophrenia, and bipolar disorder (11, 12). None of these individual features or phenotypes occurs with 100% penetrance, however, over 80% of all 22q11DS patients have some form of cardiovascular deficits and even more (>90%) exhibit

some form of cognitive and behavioral deficit. About 50% of these cardiovascular defects are classified under Tetralogy of Fallot, which consists of 4 defects (ventricular septal defects, narrowing of outflow tract, aorta that grows from both ventricles and right ventricular hypertrophy). The remaining 50% of cardiac defects are split between individuals who only have ventricular septal defects and individuals who have more severe outflow tract defects (10). The psychiatric defects range from mild ADHD or learning disabilities to severely disabling autistic spectral disorders and schizophrenia (35%). It is likely that this variability reflects a multilevel interaction with the signaling networks that provide the specification and differentiation cues to these morphogenetic domains. Thus, I have characterized molecular interactions between multiple genes in the 22q11 region and cardinal M/E signaling mechanisms in patterning the pharyngeal and forebrain morphogenetic domains.

M/E inductive signaling and its role in 22q11DS phenotypic variation

The most anterior portion of the neural tube, the telencephalon, gives rise to the cerebral cortex and its substructures including the forebrain (disrupted in 22q11DS). The development of the forebrain is governed by critical M/E interactions between signaling from the surface ectoderm, genes and signals expressed in the mesenchyme (mainly neural crest derived) and the developing neuroepithelium of the telencephalic vesicles (Figure 2). The major structural defects in the heart and aorta/outflow tract are all derived from the pharyngeal apparatus, a temporary embryological structure that is caudal and lateral to the developing head (13). It consists of a series of segmented bulges on the lateral aspect of the embryo. Each pharyngeal arch is covered externally by epithelial tissue (ectodermal in origin) and contains a mesenchymal core that consists of a

large population of neural crest cells (which delaminate from the ectodermally-derived neural tube) and endodermally-derived mesenchymal cells (Figure 3). Similar to the forebrain, pharyngeal development and morphogenesis is driven by molecular interactions between the mesenchymal and epithelial (M/E interaction) structures of the pharyngeal pouches. Thus, M/E interactions are a shared morphogenetic mechanism that might be compromised in parallel in the brain and heart in 22q11DS. I will attempt to delineate any parallel or divergent nature of these interactions between 22q11 genes and multiple signaling networks.

Clinical cardiovascular pathogenesis in 22q11DS ranges from the most frequent tetralogy of fallot (TF; 40%), truncus arteriosus (TA; 10%) and interrupted aortic arch (IA; 14%) to the less frequent double outlet right ventricle (DORV; 1%), transposition of the great arteries (1%) and patent ductus arteriosus (1%) (10, 14, 15). The pharyngeal apparatus gives rise to all the structures that are affected in 22q11DS. The pharyngeal endoderm forms the thyroid, parathyroid and thymus (16, 17) while the neural crest and cells of mesodermal lineage form the connective tissue, musculature, endothelial cells and skeletal tissue of the arch as well as its associated arteries (18-22). Multiple 22q11 genes are highly expressed in the neural crest cells as well as pharyngeal epithelium. Ablation of neural crest cells that migrate into the developing forebrain and pharyngeal region results in dysmorphology that is reminiscent of the defects seen in 22q11DS (23, 24). Additional studies show that development of the arches depends on both crest-dependent as well as crest-independent cues (25, 26). The pharyngeal endoderm has surfaced as an important source of signaling molecules (27), Hox gene expression as well as an inducer of arch morphogenesis

(28-30). Thus, it is likely that diminished dosage of a large number of 22q11 genes in 22q11DS patients may disrupt morphogenetic function and identity of either the neural crest derived mesenchyme, the surface epithelium or both, altering the mesenchymal-epithelial (M/E) communication between the pharyngeal endoderm, mesenchyme and epithelium leading to disrupted pharyngeal morphogenesis. Thus I analyzed 22q11 gene distribution within each pharyngeal compartment and examined altered pharyngeal M/E signaling in a 22q11DS mouse model.

Approximately 35% of 22q11DS patients are develop schizophrenia and a much larger percentage display other cognitive and behavioral deficits, including learning disabilities (67%), mental retardation (53%), autism (31%) and ADHD (16%) (11, 31). Given that specific regions and circuits are associated with these functions that are variably affected in 22q11DS, it is to be expected that patients with the deletion may have aberrant development of certain CNS structures that depend on M/E interactions. Indeed, children with 22q11DS seem to have altered grey and white matter volumes and exhibit enlargement of the lateral ventricles, the caudate and the insula (32). In contrast, the hippocampus and medial cerebellum are reduced in volume. There is also evidence of lateral thinning in parieto-occipital, occipital pole and inferior prefrontal regions, and medial thinning in anterior cingulate, medial frontal gyrus, subgenual prefrontal, posterior cingulate gyrus, cuneus and lingual gyrus regions in 22q11DS patients (33, 34). Few recent fMRI studies have also revealed atypical cortical activity during arithmetic and non-spatial working memory tasks (35, 36) in children with 22q11DS. Early brain development is highly integrated with the development of the face and jaw, as the same signals that pattern these external

structures also shape the development of midline patterning centers, the basal ganglia primordia and axonal inputs from the thalamus and brain stem. The cortex develops from a morphologically uniform ventricular zone located in the dorsocaudal part of the telencephalic vesicles. Cranial neural crest cells, derived from the dorsal mesencephalon and rhombencephalon, constitute a large part of mesenchymal tissue that lies adjacent to the developing brain (concentrated in anterior and ventral regions during early brain development) and acts as a signaling source. It is likely that diminished expression of 22q11 genes likely interferes with the ability of signaling centers to appropriately drive cortical morphogenesis. The range of phenotypes in 22q11DS indicates that gene dosage may be critical in these morphogenetic networks that drive remodeling of the brain. Thus, I will analyze the restricted expression of 22q11 genes in mesenchymal or epithelial compartments in the brain and heart and quantify any 22q11-gene-dosage dependent M/E signaling with in these morphogenetic domains. Together, this data will provide insight into the role of 22q11 gene dosage in signaling networks that pattern and drive the development of the brain and heart.

22q11DS phenotype: Variability and Phenocopies

Even though most 22q11DS patients have the same 3 Mb deletion, the phenotype is highly variable (10). Early specification and patterning during the development of structures affected in 22q11DS is dependent upon FGF, RA, SHH and BMPs----signals whose activity is vulnerable to disruption by genetic and epigenetic variations. The cardiovascular phenotypes in 22q11DS range from mild disruptions to life threatening anomalies. As described above, the psychiatric and behavioral manifestations range from learning disabilities to

autism, psychosis, and mental retardation. The variable severity and penetrance of the cardiovascular, psychiatric and behavioral phenotypes in 22q11DS suggests that stochastic, environmental and genetic factors outside of the deleted 22q11 locus likely modify the phenotypes. It has also been shown in mouse models that penetrance of the defects is highly dependent upon genetic background (37, 38). These findings suggest the presence of modifier genes elsewhere in the genome, perhaps on other chromosomes. Thus it is likely that signaling molecules are likely modifiers of the phenotypes.

One way to identify candidate genes is to examine mouse mutants that carry null alleles that produce phenotypically similar defects to 22q11DS. SHH, RA, FGFs, BMPs and related signaling molecules have been proposed as likely candidates. Some phenotypes in mutant mice with disrupted *Fgf8* (39) and RA (*Raldh2*) (40) signaling have been identified as 22q11DS "phenocopies", while *Shh* (41) and *Noggin*/*Chordin* (Bmp signaling) anomalies (42) are more narrowly interpreted as "parallel" to 22q11DS. It is unclear whether such similarities reflect convergence of signals and 22q11 genes consistent with the strict definition of "phenocopy", or instead, the phenotypic similarities are due to the influence of 22q11 genes and M/E signals on distinct aspects of development in similar tissues. My comprehensive expression analysis in signaling mutants and 22q11DS mouse models will assess if the relationships between M/E signals and 22q11 genes constitutes a strict phenocopy dogma or it reflects a network of interactions on multiple distinct levels.

Shh

Sonic hedgehog (SHH), a secreted, cholesterol-modified protein that binds to the transmembrane receptor Patched, activates intracellular GLI proteins,

allowing them to translocate into the nucleus and induce transcription of downstream targets (43). It is predominantly expressed in the pharyngeal endoderm in the heart and the ventral telencephalon and hypothalumus in the developing brain (44, 45). The *Shh*$^{-/-}$ mouse has a phenotype comparable to Tetralogy of Fallot with complete pulmonary atresia (41), indicating deficits in the secondary heart field, which gives rise to the outflow tract. Additional studies have shown that SHH is needed for neural crest cell survival as well as for the segmentation of the outflow tract (46). SHH-mediated proliferation in the second heart field is required for the formation of the myocardium and smooth muscle for the arterial pole and outflow tract (47). SHH is essential for the regionalization of the subpallium and also regulates morphogenesis and patterning of the pallium. Inactivation of *Shh* results in severe disruptions to midline formation and in particular for the formation of ventral brain structures (48) In addition to regulating cell fates during early neuronal specification, SHH has also been implicated in the proliferation of precursor cells in the CNS (49, 50) and granular cell precursors in the cerebellum during late embryogenesis (51-53). Thus, SHH's important roles in processes that appear to be disrupted in 22q11DS (secondary heart field development and ventral pallial morphogenesis) indicate that the signal and several 22q11 genes may be involved in the same morphogenetic networks.

RA

During development Retinoic Acid (RA) serves as a ligand for nuclear receptors that regulate developmentally important genes. It influences the pattern formation of several structures including the brain and the heart (54-56). RA is primarily synthesized in the neural crest derived mesenchyme in the

pharyngeal apparatus and the ventrolateral mesenchyme of the developing forebrain (56, 57). Loss or gain of RA signaling by either genetic means or exposure to exogenous RA has severe consequences to embryonic development. Deletion of an RA synthetic enzyme *Raldh2* results in a heart tube that fails to loop or form any posterior compartments. Mice carrying one hypomorphic allele and one null allele (*Raldh2*$^{neo/-}$) display 22q11DS related thymic, parathyroid and cardiovascular defects including a persistent truncus arteriosus (PTA), ventricular septal defect (VSD) and aortic arch artery remodeling defects (40). Ablation of the RA receptors (RARa and RARb) results in exencephaly, underdeveloped telencephalic vesicles and abnormal frontonasal development (58). Deletion of a RA metabolizing enzyme, *Cyp26a1*, results in severe posteriorization of the embryo and disrupts rhombomere identity and in turn neural crest cells that contribute to forebrain development (59). Given its role in the development of the forebrain and heart structures that are also disrupted in 22q11DS, and its activity in 22q11-gene expressing tissue, it is likely that RA may interact with 22q11 genes during cardiovascular and forebrain morphogenesis.

FGF's

FGF8, a receptor-tyrosine-kinase signaling molecule, has a number of roles throughout heart and brain development. In the heart it is expressed in the pharyngeal endoderm and signals to the adjacent pharyngeal mesoderm and in the developing brain it is expressed at the rostral margin of the telencephalon. *Fgf8*-null mice have early heart looping abnormalities (60) while the *Fgf8* hypomorphs develops double outlet right ventricle, persistent truncus arteriosus, and has disrupted pharyngeal arch artery patterning (39). Thus, heart development is sensitive to FGF8 dosage. In *Fgf8* deficient embryos, 22q11-gene

expressing cardiac neural crest cells die en route to and within the pharyngeal arches. Thus, FGF8 is necessary for 22q11-gene expressing neural crest cells to reach the pharyngeal arches and function normally in the septation of outflow tract (61). There is similar dose-dependency in the developing brain. FGF8 promotes telencephalic outgrowth and regulates its rostral regionalization. FGF8 dosage appears to mediate the size and nature of the neocortex as well as telencephalic midline structures and basal ganglia (62-66). Together, these data suggest that FGF signaling is important for processes that appear to be disrupted in 22q11DS such as the addition of outflow tract myocardium by 22q11-gene expressing cardiac neural crest cells from the secondary heart field and the organization of the developing forebrain.

BMP's

In both the heart and the brain, BMPs and WNTs seem to co-ordinate proliferation and differentiation of progenitor cell populations (67, 68). Two important mediators of BMP signaling, Chordin and Noggin, are expressed in the dorsal endoderm and roofplate respectively (42, 69). Genetic ablation of Chordin alone, or in conjunction with Noggin, induces severe cardiovascular as well as cranial and spinal cord deficits in early gestation embryos (42). There is early expansion of the ventral mesoderm indicating the need for appropriate Bmp signaling for normal dorso-ventral patterning. The Chordin null lacks a thymus and parathyroid and has a hypoplastic thyroid (all derivative of the 3rd and 4rth pharyngeal pouch). In addition, the mutant exhibits persistent truncus arteriosus and do not form any of the pharyngeal arch arteries (42). Chordin; Noggin double mutant embryos lack extensive regions of the forebrain, as well as eyes, nasal placodes and facial structures. In addition, the telencephalon and

diencephalon are reduced to a small vesicle of thin neuroepithelium. In contrast, more posterior brain structures are relatively normal (70). Thus, appropriate antagonism and control of BMP signaling is required for the normal development of the forebrain as well as the pharyngeal structures that are disrupted in 22q11DS.

22q11 genes: the search for a candidate

Expression of 22q11 genes is spatially and temporally coincident with the activity of the 4 signaling networks described above. In order to understand the contribution of 22q11 genes to the complex network of cardinal-signal-driven development events, several mouse models have been generated, with each carrying deletions of varying lengths in the murine genome orthologous to the 22q11.2 region in the human (*LgDel(71)*; Df1(72); Figure 1). The defects in these mice closely recapitulate the cardiovascular phenotypes seen in human 22q11DS patients. Some of these vascular defects are partially rescued by crossing the *LgDel* mice with mice carrying 1-2 copies of artificial BAC's containing 3-4 human genes from the 22q11 region. Complementing the deletion with one particular BAC (BAC 316; Figure 1) containing *Gnb1l*, *Tbx1*, *Septin5* and *Cldn5* (2 structural proteins, 1 transcription factor and 1 protein of unknown function) reduces the penetrance of vascular defects from ~45% to ~14%, indicating that these 4 genes are at least partially responsible for the cardiovascular defects seen in 22q11DS (71). Additional analysis of *Tbx1* has shown localization to the pharyngeal epithelium. The varied penetrance (19%-43%) of pharyngeal arch defects in $Tbx1^{+/-}$ embryos has further implicated it in the development of cardiovascular defects (73). The lack of complete rescue by complementation

indicates that it is likely that additional genes within the 1.5Mb region contribute to the cardiovascular defects.

To identify additional contributors to the phenotypes, several single-gene as well as contiguous gene knockouts have been made. Knockdown of another 22q11 gene *Dgcr6* results in cardiovascular defects in chicken (74) in addition to altering expression of multiple other 22q11 genes (*Tbx1*, *Ufd1l* and *Hira* --- genes potentially involved in cell cycle). Conditional ablation of a mitochondrial metabolic gene, *Txnrd2,* disrupts cardiomyocyte proliferation and results in fatal dilated cardiomyopathy (75). While several reports have claimed that *Tbx1* alone is responsible for the heart phenotypes (71, 76, 77), closer examination of the above data and retrospective human genetic screens indicate that loss of *Tbx1* only partially contributes to the cardiovascular phenotype. *Dgcr6*, *Txnrd2* and *Ufd1L* (and possibly *Hira*) potentially also contribute to the cardiovascular phenotype seen in 22q11DS by disrupting M/E signaling and associated cellular processes that are critical for pharyngeal morphogenesis. It is likely that disruption of many genes of varying function (cell cycle, transcription, metabolism, nucleic acid processing, structural) within the region disrupt signaling and morphogenesis to a greater degree than just one gene alone. Thus, diminished dosage of multiple 22q11 genes, such as but not limited to, *Dgcr6*, *Txnrd2*, *Ufd1l*, *Tbx1* and *Hira* may disrupt cell-cell communication between the pharyngeal mesoderm and pharyngeal endoderm resulting in significant variability in development of the musculature, connective tissue and endothelial structures of the developing heart.

It is likely that diminished dosage of one or more 22q11 genes disrupts neurogenesis, specification, differentiation or synaptogenesis leading to

structural as well as functional CNS deficits seen in 22q11DS. The search for genes contributing to the psychiatric phenotypes in 22q11DS has lead to a number of candidates, including *Comt* (a metabolic protein involved in the key pathways for the degradation of catecholamines), *Dgcr8* (part of the micro-RNA processing complex), *Prodh* (proline metabolism), *Rtn4r*/*NoGoR* (a cell surface protein potentially involved in axon pathfinding) and *Arvcf* (Catenin family member involved in surface cell-cell interaction; potentially involved in Wnt signaling). *Comt* is a critical component of the pathway that degrades catecholamines including dopamine and epinephrine. Delayed or disrupted metabolism of these catacholamines from synapses could impair brain function due to elevated levels of these neurotransmitters (78). *Dgcr8*, is an integral part of a microprocessor machinery that is required for miRNA production and heterozygous loss leads to downregulation of a subset of mature miRNA's (79). Diminished expression of a microRNA gene contained within the minimal deleted 22q11 region (mir-185) could have significant effects on the stability of target transcripts. While specific targets of mir-185 have not been established it is likely that altering miRNA levels during development due to deletion of *Dgcr8* and mir-185 could disrupt expression of developmentally critical genes and lead to disrupted cortical architecture and 22q11DS phenotypes. Diminished expression of *Prodh*, responsible for metabolism of L-proline, an amino acid that affects GABA-ergic neurotransmission, is responsible for increase levels of L-proline found in about 50% of individuals with 22q11 deletion. Increased levels of this amino acid has been linked to epilepsy (80) and schizoaffective disorder (81, 82). Thus it is likely that diminished expression of *Comt*, *Dgcr8*, *Prodh* and several others within the 22q11.2 region (*Txnrd2*, *Arvcf*, *NoGoR*, *mir-185*) could

significantly disrupt cortical circuitry and architecture leading to a high incidence of psychiatric disorders seen in 22q11DS patients. All these prior studies have taken the initial step of understanding the physiological contribution of some individual genes, but 22q11DS is the consequence of a multigenic deletion and must be studied as such. Understanding the developmental and physiological implications of a combined reduction in dosage of more than 30 genes will allow us to truly discern the molecular basis for 22q11DS. Hence I will undertake a comprehensive analysis of the interaction of the 22q11.2 region and M/E signaling mechanisms that drive early morphogenetic events of the heart and brain.

Interaction of signaling networks and 22q11 genes

If multiple 22q11 genes and the cardinal M/E signals that pattern the forebrain and pharyngeal morphogenetic domains interact to facilitate appropriate cell-cell communication and in turn normal morphogenesis, then disrupting both should lead to new phenotypes not explained by additive effects alone. Appropriate morphogenesis of embryonic structures is regulated by the temporal and spatial integration of signals (BMP, SHH, RA and FGF) that modulate cellular proliferation, survival and fate. The secreted signaling molecules generated from a mosaic of patterning centers govern CNS as well as cardiovascular development. Most BMPs are expressed in the dorsal prosencephalon and dorsal endoderm (68), whereas SHH is ventromedially restricted in the brain and in the pharyngeal endoderm (83, 84), RA is expressed ventrolaterally in both regions (57) and FGF8 is expressed rostrally in the developing prosencephalon and in the endoderm adjacent to the precardiac mesoderm (85). These signaling molecules define dorsoventral and

anterioposterior axes. In addition, expression of inhibitors and receptors for each signal create a more complex mosaic of signaling within each morphogenetic domain. The existence of signaling centers in each structure where distinct regions express BMP, SHH, RA and FGF sets up an interactive signaling environment where the individual and combined actions of these cardinal signals drives appropriate morphogenesis. Thus, temporally altering this carefully controlled pattern of signaling in *LgDel* mice by ectopic administration of signaling agonists and antagonists will provide insight into developmental processes that rely on interactions between the deleted region and the altered M/E signal.

Several studies in the developing brain, pharyngeal regions and limb (all regions affected in 22q11DS) indicate that there is significant interaction between these signaling pathways, (44, 86-90). In addition to interacting with each other, these signaling morphogens pattern expression of homeobox genes and important transcription factors in the developing brain and heart (44, 91). Overlap of phenotypes in signaling mutants and 22q11DS mouse models indicate that there might be interaction of 22q11 genes with in these signaling networks. Indeed, some initial evidence shows that several signaling molecules regulate expression levels and patterns of a 22q11 gene, *Tbx1 (92)* and that there might be some interaction between multiple 22q11 genes and developmental signals *(93-96)*. Taken together, this data indicates that it is more appropriate to generate a comprehensive and integrative model of signaling gene networks and the role of 22q11 genes within them. My experiments will attempt to clarify the relationship between 22q11 genes and cardinal signals as either 'downstream'

(regulated by M/E signals) or 'upstream' (influencing M/E signaling) by delineate the regulatory as well as interactive nature of this relationship.

Gene dosage: Morphogenetic thresholds and variability

Congential heart and brain defects have significant variability that is not explained by total loss of one gene alone. 22q11DS accounts for 15% of patients with tetralogy of Fallot (TF), and over 50% of children with interrupted aortic arch (IAA) and truncus arteriosus (TA) (97). 22q11DS patients also account for 8% of children with cleft palate, submucous cleft palate and occult submucous cleft palate (98, 99). There is also a high degree of overlap in phenotypes with other etiologically diverse multiple-anomaly genetic and epigenetic disorders (fetal alcohol syndrome (100), retinoic acid teratogenesis *(101, 102)*, Downs syndrome (103), Rett syndrome (104), Noonan syndrome (105), Fragile X (106); Figure 4). While 22q11DS patients exhibit the full gamut of heart phenotypes, patients suffering from Fetal Alcohol Syndrome and Downs Syndrome exhibit TF and some septal defects while Noonan Syndrome patients have less severe septal defects and Fragile-X patients do not exhibit any abnormal phenotypes of the heart. Cognitive deficits in 22q11DS patients range from mild behavioral deficits to high incidence of ADHD, autism, mood disorders, and schizophrenia. None of the other single gene mutation or aneuploid diseases above have a high incidence of schizophrenia but share increased incidence of ADHD, autism and intellectual disability with 22q11DS. The partial penetrance and partial recapitulation of the phenotype in single gene mutants underscore the importance of studying 22q11DS and other multigenic diseases in a more integrated fashion. Understanding the molecular interaction between genes deleted in 22q11DS and cardinal signaling networks that drive early

morphogenesis of the structures affected in 22q11DS will shed light on potential etiology of similar defects that occur more commonly in the general population.

Our multifaceted approach to studying these interactions has shown that 22q11 genes are primarily expressed in mesenchymal tissue at sites of M/E interaction (i.e the developing forebrain and pharyngeal arches) and that their expression is critically dependent upon Shh and to a lesser extent RA. Heterozygous loss of the entire 22q11.2 region, not just *Tbx1* alone, specifically alters local levels of Shh and RA signaling. In addition we find that 22q11 genes establish a dynamic range of Shh and RA signaling variation within which morphogenesis can proceed normally. This range is constricted by 22q11 deletion leading to significant dysmorphogenesis of brain and pharyngeal structures in the face of signaling variation. Thus, our data suggests that morphogenesis of the brain and pharyngeal apparatus depends upon coordinate expression and interaction between 22q11 genes and signaling mediators in mesenchymal and epithelial tissues. Loss of 22q11 gene expression, primarily in the mesenchyme, likely alters the ability of this tissue to sustain appropriate morphogenesis in the face of signaling volatility leading to significant phenotypic variability in 22q11DS patients.

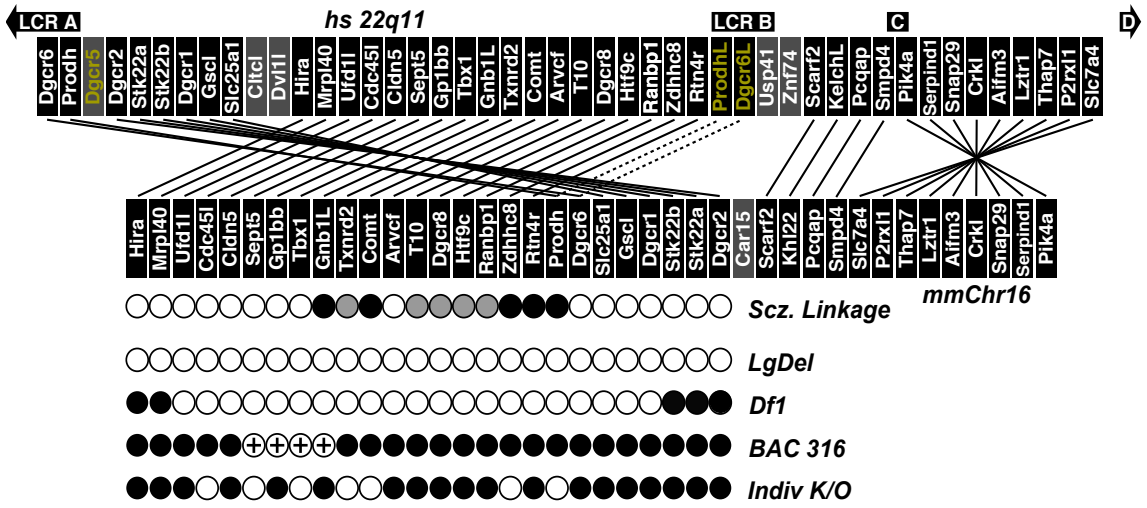


Figure 1: Gene map of the 22q11.2 region and its orthologous region on the mouse chromosome 16. 3Mb deletion is between LCR-A and LCR-D. Nested 1.5MB deletion between LCR-A and LCR-B. Linkage of several individual genes to schizophrenia. *LgDel* mice have deletion of 26 genes. *Df1* mouse has a nested 21 gene deletion. BAC 316 used in complementation studies contains additional copies of 4 genes (indicated with a +). Individual mouse knockouts that have studied in the literature.

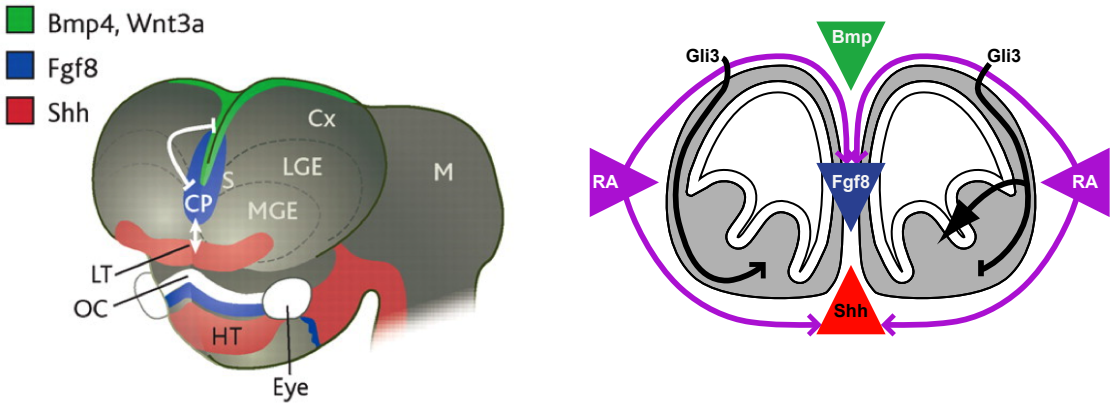


Figure 2: Mosaic of M/E signaling centers in the developing brain

*Adapted from Ohkubo etal 2002 (44) and Tucker et al (107)

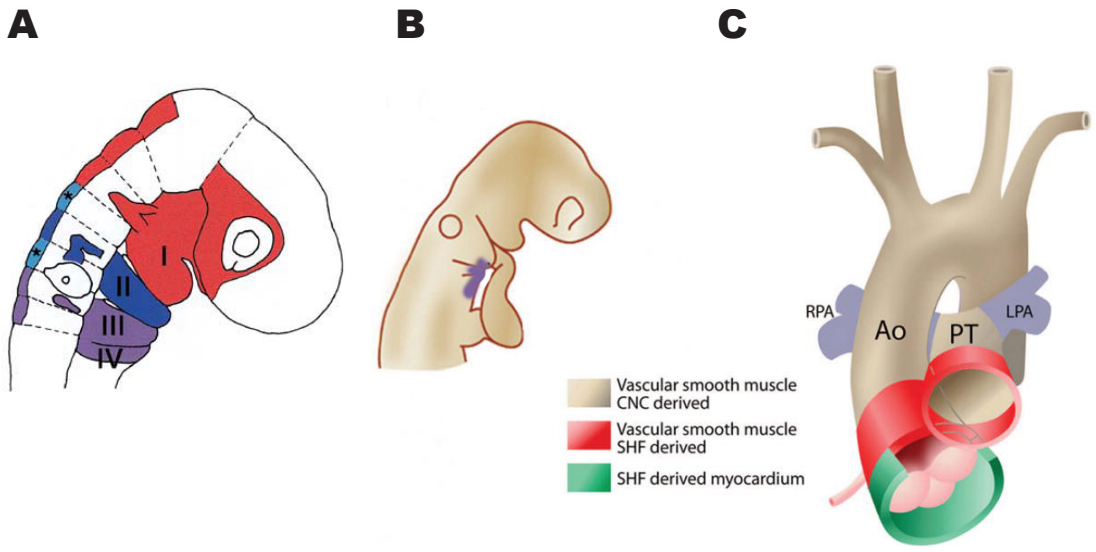


Figure 3: Formation of the arterial pole (A) Cardiac neural crest (purple – from rhombomeres 5/6) populate the second heart field. (B) Origin of the secondary heart field cells (purple) in the ventral pharynx (C) Myocardium (green) and smooth muscle (red) derived from the secondary heart field that form the arterial pole.

*Adapted from Hutson and Kirby 2007 (108)

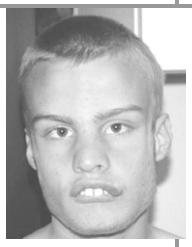
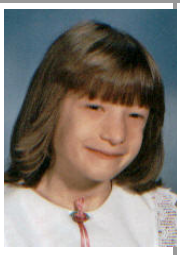
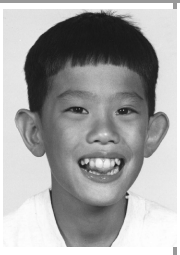
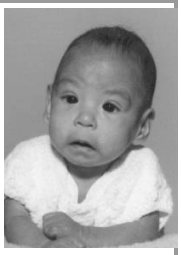
		Autism (microdeletion)	Down syndrome (Trisomy 21)	VCFS (22q11 microdeletion)	Fragile X (FMRP mutation)	Rett syndrome (MECP2 mutation)	Fetal Alcohol Syndrome
							
		Jacquemont et al. 2006	dsahr.org	cardiogenetics.org	genetics.utah.edu	rettsyndromealberta.org	wikipedia.org
	Estimated incidence	1:500	1:1000	1:4000	1:4000 (male) – 1:6000 (female)	1:12,500 (females)	1:750
Anomailes	Cognitive	Yes	Yes	Yes	Yes	Yes	Yes
	Psychiatric	Yes	Alzheimers (~25% by age 35)	Schizophrenia (25%) Autism (20%)	Autism (~20%)	Autistic-like Progresses to MR	ADHD, Depression, others (90%)
	Craniofacial	Yes	Yes	Yes	Yes	Yes	Yes
	Cardiac	Not noted	Yes (VSD)	Yes (VSD)	Yes (Mitral Valve)	Arrythmyias (No structural)	Yes (VSD)
	Limb & digit	Yes	Yes	Yes	Yes	Yes	Yes

Figure 4: Overlapping phenotypes of etiologically diverse genetic and epigenetic disorders

CHAPTER 2

INTRODUCTION - II

22q11 deletion syndrome (22q11DS), also known as DiGeorge or Velocardiofacial Syndrome, is the consequence of a hemizygous loss of a "critical" (1.5Mb) or larger "typical" (3MB) region of human Chr.22 (6, 109). 22q11DS phenotypes, seen with variable penetrance, include life-threatening cardiovascular malformations (2, 10), craniofacial and limb abnormalities (110), parathyroid and thymic hypoplasia (111), and increased susceptibility to behavioral disorders and psychiatric diseases (112) suggesting altered brain development or function. Initial differentiation of most affected structures, including the brain and heart, depends upon mesenchymal/epithelial (M/E) interactions, mediated by signaling via Sonic Hedgehog (Shh), Fibroblast Growth Factors (Fgfs), Retinoic Acid (RA), and Bone Morphogenetic Proteins (Bmps) (113, 114). Loss-of-function mouse models for these M/E signals have phenotypes that overlap, to varying degrees, those in 22q11DS. Coincident expression and activity of 22q11 genes, Shh, Fgfs, RA and Bmps at M/E sites—including the limb, face, heart and brain—suggest that normal signaling may be disrupted by altered 22q11 gene dosage (115). Nevertheless, it remains unknown whether 22q11 genes interact significantly with the cardinal M/E signals. Thus, we asked whether Shh, Fgf, RA, and Bmp signaling influences 22q11 gene expression and activity as well as whether diminished 22q11 gene dosage

modifies these signaling pathways during mid-gestation, when M/E signaling initiates significant morphogenesis at sites compromised in 22q11DS.

Variable severity and penetrance of 22q11DS phenotypes suggests modifiers outside of the deleted 22q11 locus, and Shh, Fgfs, RA, Bmps, and related signaling molecules are likely candidates. Some phenotypes in mutant mice with disrupted *Fgf8* (39) and RA (*Raldh2*)(40) signaling have been identified as 22q11DS "phenocopies", while *Shh* (41) and *Noggin*/*Chordin* (Bmp signaling) anomalies (42) are more narrowly interpreted as "parallel" to 22q11DS. It is unclear whether such similarities reflect convergence of signals and 22q11 genes consistent with "phenocopy", or influence of 22q11 genes and M/E signals on distinct aspects of development in similar tissues. Shh and RA apparently regulate expression of at least one 22q11 gene, *Tbx1* (92, 94) which when deleted in combination with *Crkl* (outside of the 1.5MB critical region, but within the broader 3MB "typical" region;(6, 116)) can modulate RA and *Fgf8* signaling (93), while total loss of *Tbx1* down-regulates *Bmp4* (76). Moreover, recent observations suggest that additional genes outside the 22q11 minimal region modulate *Tbx1* mutant phenotypes (117, 118), some of which may be associated with RA (117) and Bmp (119) signaling. These observations, however, do not address interactions with 22q11 genes beyond *Tbx1*, which although critical, may not explain the full range of 22q11DS phenotypes (93, 115, 120, 121). Thus, we asked if M/E signals regulate 22q11 genes in the critical region; whether dosage of these genes influences Shh, Fgf, RA or Bmp signaling, and whether phenotypes in 22q11DS mouse models can be modified by altered inductive signaling.

We found clear quantitative evidence of significant interaction between 22q11 gene dosage and Shh as well as RA signaling pathways. Moreover, disrupted

Shh and RA signaling in mouse embryos with a deletion parallel to that in 22q11DS results in significantly more severe phenotypes than altered signaling, 22q11 deletion or heterozygous *Tbx1* deletion alone. The reciprocal regulation and phenotypic modulation we have found does not support simple, linear, uni-directional relationships between cardinal M/E signals and 22q11 genes; instead, they are likely part of a broader network that modifies distinct aspects of M/E-mediated morphogenesis in the heart, brain and other phenotypic sites. This network may be an essential contributor to the phenotypic variability seen in 22q11DS patients.

CHAPTER 3

RESULTS

Shh, BMP, FGF and RA signaling influence 22q11 gene expression

The 21 mouse orthologues of genes within the 22q11 critical region known to be selectively or specifically expressed at sites of M/E interaction (122) are selectively expressed in the mesenchymal compartment (Figure 5A; [21]). Analysis of the developing forebrain and branchial arches show that with the exception of 3 epithelium-restricted genes (3/21; *Tbx1*, *Gnb1L* and *Slc25a1;* Figure 5A) the majority of 22q11 genes (18/21) are exclusively or selectively expressed in the mesenchyme. Heterozygous deletion of 28 contiguous murine 22q11 orthologues (including 7 not expressed in the embryonic or adult brain, and thus not analyzed further here;(122)) in embryonic (E)10.5 *LgDel* embryos (71) results in a 50% expression decrement for each corresponding mRNA (Figure 5B), presumably primarily within the mesenchyme, where most of these genes are highly expressed. Thus, we asked whether altered Shh, RA, Fgf8, or Bmp signaling at E10.5, the peak age when each of these signals mediates M/E interactions at 22q11DS phenotypic sites—limbs, heart, face and forebrain—disrupts expression of any 22q11 gene, perhaps to levels that seen following heterozygous deletion Dysmorphology and tissue loss complicates analysis at sites of M/E induction in *Shh*, *Raldh2* (RA), *Fgf8*, or *Noggin* (Bmp) homozygous

mutant embryos. To circumvent this issue, we also manipulated each signal using pharmacological antagonists or agonists between E9.5 and E10.5.

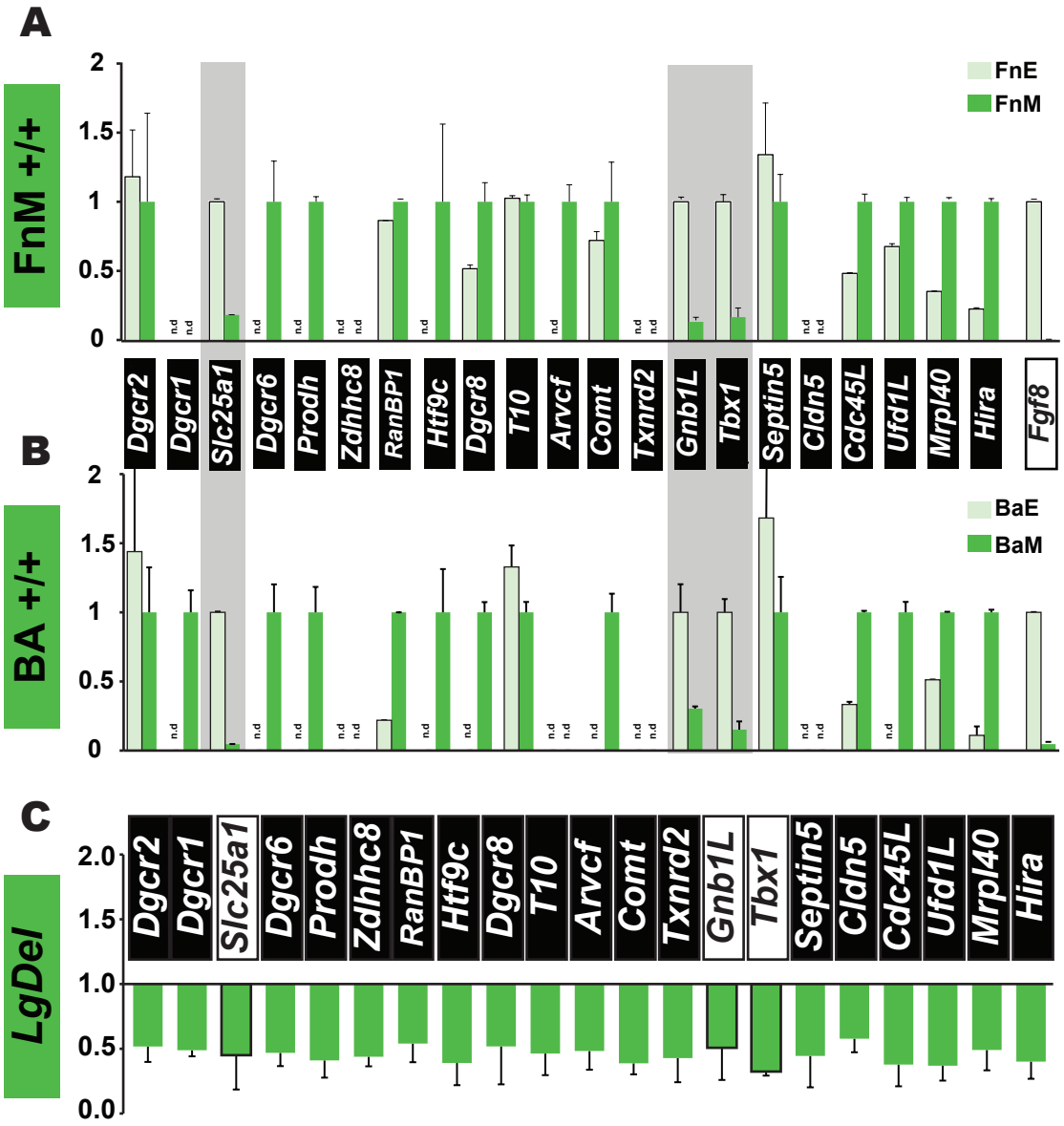


Figure 5: 22q11 genes are expressed primarily in the mesenchyme at sites of M/E interaction, and are diminished by 50% by heterozygous deletion. (A) Tissue specific mRNA expression of twenty-one 22q11 genes in the epithelium and mesenchyme of the frontonasal mass (FnM; n=3). 11/17 detected genes are restricted to mesenchyme while 3 (grey shaded box) are restricted to the epithelium. The remaining 3 are expressed equally in mesenchyme and epithelium. (B) Tissue specific expression of the same 22q11 genes in Branchial Arch 1 (BA; n=3). Detection and tissue-specific expression mostly mirror that in the FnM; however, *Dgcr1* is detected only in the BA mesenchyme while *Arvcf* is detected only in the FnM-mesenchyme. (C) Twenty-one 22q11 genes are expressed at 50±10% of wild-type levels following genetic heterozygous loss of 22q11 genes in the *LgDel* embryo (n=3, $p \leq 0.05$; 2-tailed T-test). Mesenchymal or dual expressed genes indicated in black boxes with white letters; epithelial restricted genes are indicated in white boxes with black letters.

Shh regulation of 22q11 gene expression

Expression of ten of twenty-one highly expressed 22q11 genes was significantly altered by constitutive loss of Shh signaling in E10.5 *Shh*$^{-/-}$ embryos. Two of these ten are epithelially restricted (Figure 6A, thick outlined bars) while the remaining 8 are preferentially expressed in the mesenchyme. In mRNA samples from individual whole *Shh*$^{-/-}$ mutant embryos (n=4), seven of the ten genes that have altered expression are reduced to 50% of wild type levels (Figure 6A, solid bars; comparisons based upon one way ANOVA for wild-type, *LgDel*, and *Shh*$^{-/-}$ embryos, $p \leq 0.05$), paralleling that in the *LgDel* embryo (*LgDel*-like). Three additional genes are either reduced beyond 50% or significantly increased (Figure 6A, hatched bars; $p \leq 0.05$). Of the seven genes that have *LgDel*-like expression changes, two are restricted to the epithelium (Figure 6A, thick outlined bars) and the other five are mesenchymal. Analysis of the *Gli3*$^{Xtj/Xtj}$ embryos (a presumed gain of Shh function;(45)) did not show reciprocal changes of 22q11 genes, *Fgf8,* or *P75* (Figure 6B).

mRNA levels for epithelial (*Fgf8;* (85)) and mesenchymal markers (*P75*; (123)) are also significantly diminished in *Shh*$^{-/-}$ embryos, complicating interpretation of parallel diminished levels of 22q11 genes. Accordingly, to assess acute effects of Shh signaling, independent of concatenated morphogenetic disruption at M/E sites in *Shh*$^{-/-}$ embryos, we reduced Shh signaling between E9.5-E10.5 via maternal exposure to cyclopamine, which blocks *Shh* signal transduction (124) without the dysmorphogenesis seen in *Shh*$^{-/-}$ embryos. Brief, transient cyclopamine exposure results in a 50% reduction of *Ptch1*, a known Shh target gene (125) (Figure 6D); however, neither *Fgf8* nor *P75* are diminished.

Cyclopamine-dependent loss of Shh signaling also significantly alters expression of ten 22q11 genes. Eight of these ten genes are diminished by 50%, thus paralleling *LgDel* (Figure 6C; solid bars; $p \leq 0.05$), while an additional two are either increased, or reduced by less than 50%. Three of these eight genes are restricted to the epithelium (Figure 6C; solid yellow bars with thick outline), overlapping *Shh* expression domains. Five genes: *Slc25a1*, *Dgcr6*, *Arvcf*, *Tbx1*, and *Septin5* are reduced to *LgDel* values in cyclopamine-treated as well as $Shh^{-/-}$ embryos.

We next assessed whether Shh signaling regulates 22q11 genes by maintaining normal expression levels or patterns using *in situ* hybridization (ISH) to analyze genes that substantially decrease or increase in response to genetic and pharmacological manipulation of Shh signaling. We evaluated *Sept5,* which is significantly diminished by both $Shh^{-/-}$ and cyclopamine, as well as *Ranbp1,* which is significantly increased by cyclopamine. For both genes, ISH labeling in cyclopamine-exposed embryos indicates diminished (*Sept5*) or enhanced (*Ranbp1*) levels, but not patterns of wild-type expression at sites of M/E induction (Figure 6E; n=4 for each probe). Thus, Shh signaling likely maintains normal levels, without apparent change in pattern, of a substantial subset of 22q11 genes in the mesenchyme and epithelium at 22q11DS phenotypic sites.

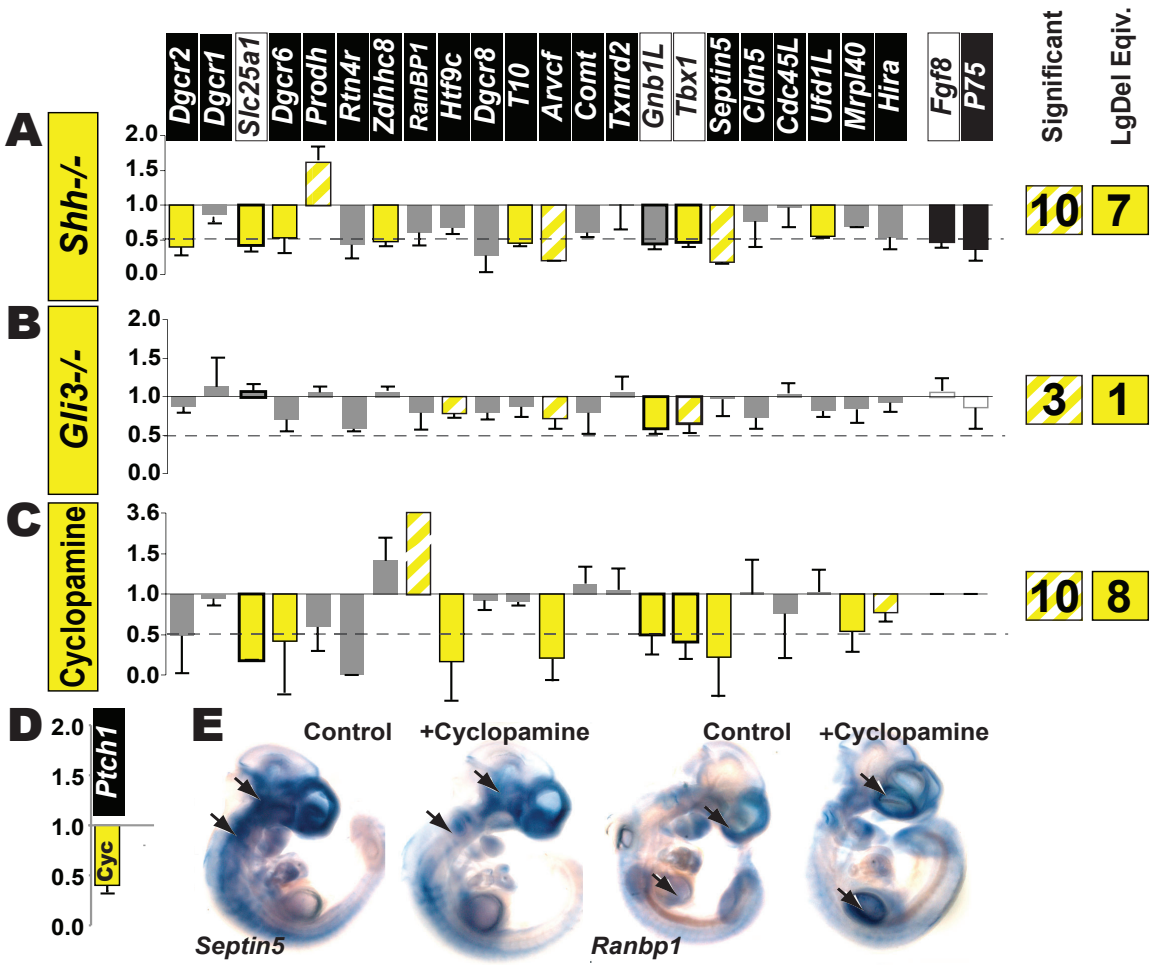

Dgcr2
Dgcr1
Slc25a1
Dgcr6
Prodh
Rtn4r
Zdhhc8
RanBP1
Htf9c
Dgcr8
T10
Arvcf
Comt
Txnrd2
Gnb1L
Tbx1
Septin5
Cldn5
Cdc45L
Ufd1L
Mrpl40
Hira
Fgf8
P75
Significant
LgDel Eqiv.
A
Shh-/-
10
7
B
Gli3-/-
3
1
C
Cyclopamine
10
8
D
Ptch1
Cyc
E
Control
+Cyclopamine
Control
+Cyclopamine
Septin5
Ranbp1

Figure 6: Sonic hedgehog (Shh) signaling maintains normal levels of a substantial number of 22q11 genes. Epithelium-restricted 22q11 genes are indicated by thicker black outlines on bars. (A) Expression of ten 22q11 genes (yellow bars, hatched or solid) changes significantly in E10.5 *Shh*$^{-/-}$ embryos (n=4; p≤0.05; t-test); 7 of these changes are statistically equivalent to changes in the *LgDel* (solid yellow bars; p≥0.05; 2-way ANOVA) embryo. Significantly decreased expression of epithelial *Fgf8* and mesenchymal *P75* (black) suggests altered integrity of the respective tissues. (B) Minimal expression changes of 22q11 genes in E10.5 *Gli3*$^{-/-}$ embryos (4/22 genes, hatched yellow bars); without significant changes in *Fgf8* or *P75* (open bars). (C) Transient cyclopamine-induced inhibition (24-hr exposure; see Methods) of Shh signaling decreases expression of 10 22q11 genes (n=4, p≤ 0.05, t-test). Hatched bars indicate significant changes not equivalent to *LgDel*; 8 solid yellow bars are equivalent to *LgDel* (n=4; p≥0.05; 2-way ANOVA). Neither epithelial *Fgf8* nor mesenchymal *P75* expression is altered. (D) *Ptch1*, a known Shh target gene is downregulated to 50% of wild-type levels following 24-hr cyclopamine treatment (p≤0.05; n=4). (E) Changes in apparent intensity, but not pattern, of expression of *Septin5* and *Ranbp1* (largest magnitude changes) in response to 24-hr cyclopamine exposure shown by *ISH* in E10.5 embryos (n=5 for each probe).

RA signaling and regulation of 22q11 gene expression

Disrupted RA signaling in *Raldh2*$^{-/-}$ embryos (54) is accompanied by significantly decreased expression of sixteen 22q11 genes. Fifteen of these expression changes are statistically equivalent to changes seen in *LgDel* embryos (Figure 7A; solid bars; $p \leq 0.05$). All but one gene is restricted to the mesenchyme, overlapping with domains of RA activity. These changes, however, are paralleled by significant expression changes of *Fgf8* and *P75* (50% and 200% respectively; Figure 7A), and likely reflect severe morphogenetic consequences, especially for mesenchyme, of constitutive loss of *Raldh2* function at M/E sites (40). To circumvent interpretative difficulties due to likely tissue loss versus altered 22q11 gene expression, we briefly enhanced or diminished RA signaling between E9.5 and 10.5 using *all trans* RA ((126); Figure 7B) and DEAB (an RA synthesis inhibitor; (127); Figure 7C), both of which alter expression of RA-regulated genes including *Rar*α (126), but do not significantly change *Fgf8* or *P75* (Figure 7B,C). RA significantly diminishes three genes—*Ranbp1*, *Gnb1l*, and *Cdc45l*—none, however, by 50% or more (Figure 7B, $p \leq 0.05$). In contrast, RA increases *Prodh* and *Septin5* (Figure 7B, 50-60%, $p \leq 0.05$). These changes indicate that our sub-teratogenic RA dosing regimen is the least disruptive of all our pharmacological manipulations. DEAB diminishes expression of six mesenchymal genes—*Dgcr2*, *Dgcr6*, *Dgcr8, Septin5, Cldn5 and Hira*—to *LgDel* levels (Figure 7C, $p \leq 0.05$). ISH for *Ranbp1* and *Dgcr8*, which are significantly decreased by RA and DEAB treatments respectively, indicates these changes reflect local fluctuations in expression level rather than pattern (Figure 7D,E). Apparently, RA signaling

modestly influences 22q11 gene expression levels at M/E inductive sites compromised in 22q11DS; however, there is no clear relationship between presumed increased (+RA) and decreased (*Raldh2*$^{-/-}$ or DEAB) signaling.

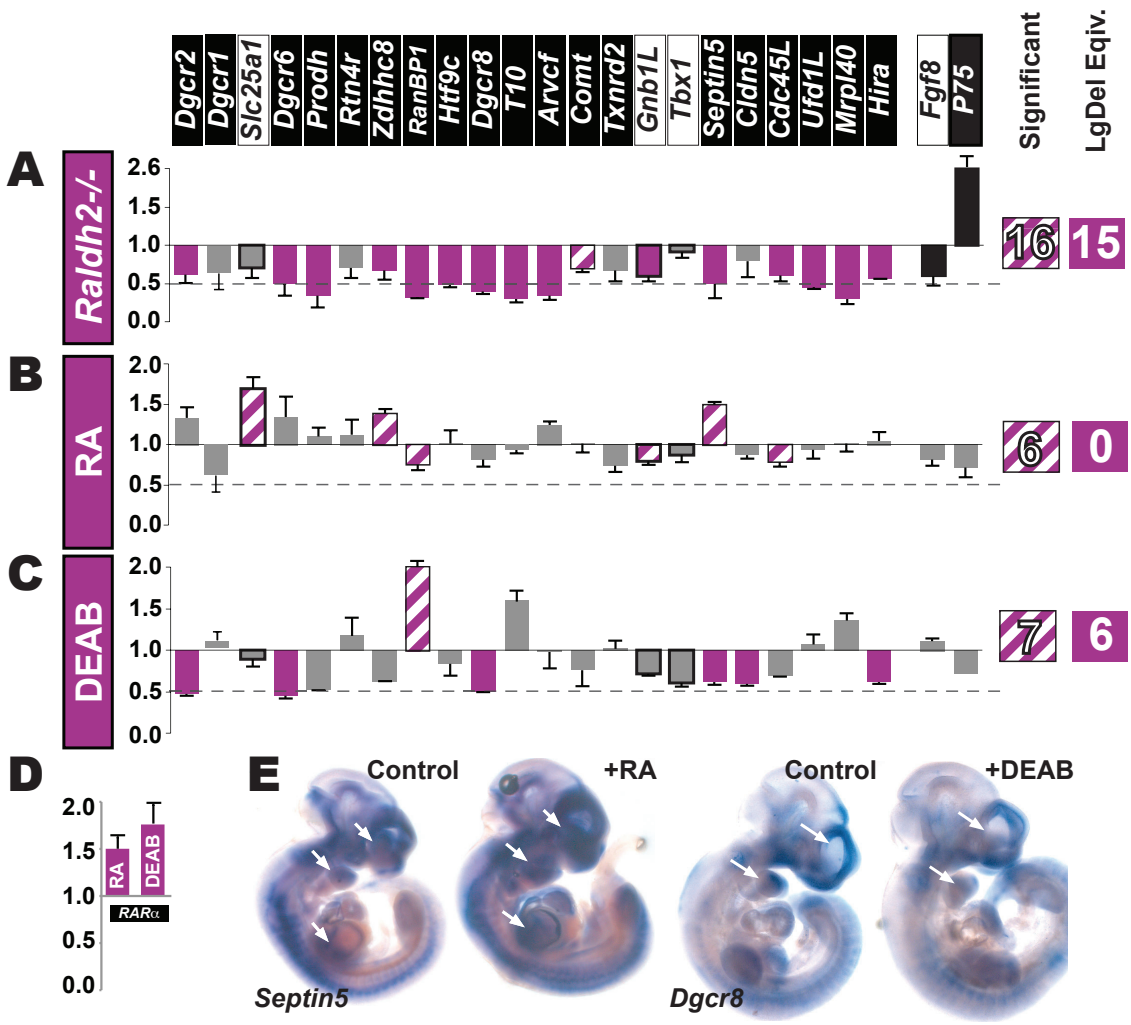

Dgcr2
Dgcr1
Slc25a1
Dgcr6
Prodh
Rtn4r
Zdhhc8
RanBP1
Htf9c
Dgcr8
T10
Arvcf
Comt
Txnrd2
Gnb1L
Tbx1
Septin5
Cldn5
Cdc45L
Ufd1L
Mrpl40
Hira
Fgf8
P75
Significant
LgDel Eqiv.
A
Raldh2-/-
2.6
1.5
1.0
0.5
0.0
16
15
B
RA
2.0
1.5
1.0
0.5
0.0
6
0
C
DEAB
2.0
1.5
1.0
0.5
0.0
7
6
D
2.0
1.5
1.0
0.5
0.0
RA
DEAB
RARα
E
Control
+RA
Control
+DEAB
Septin5
Dgcr8

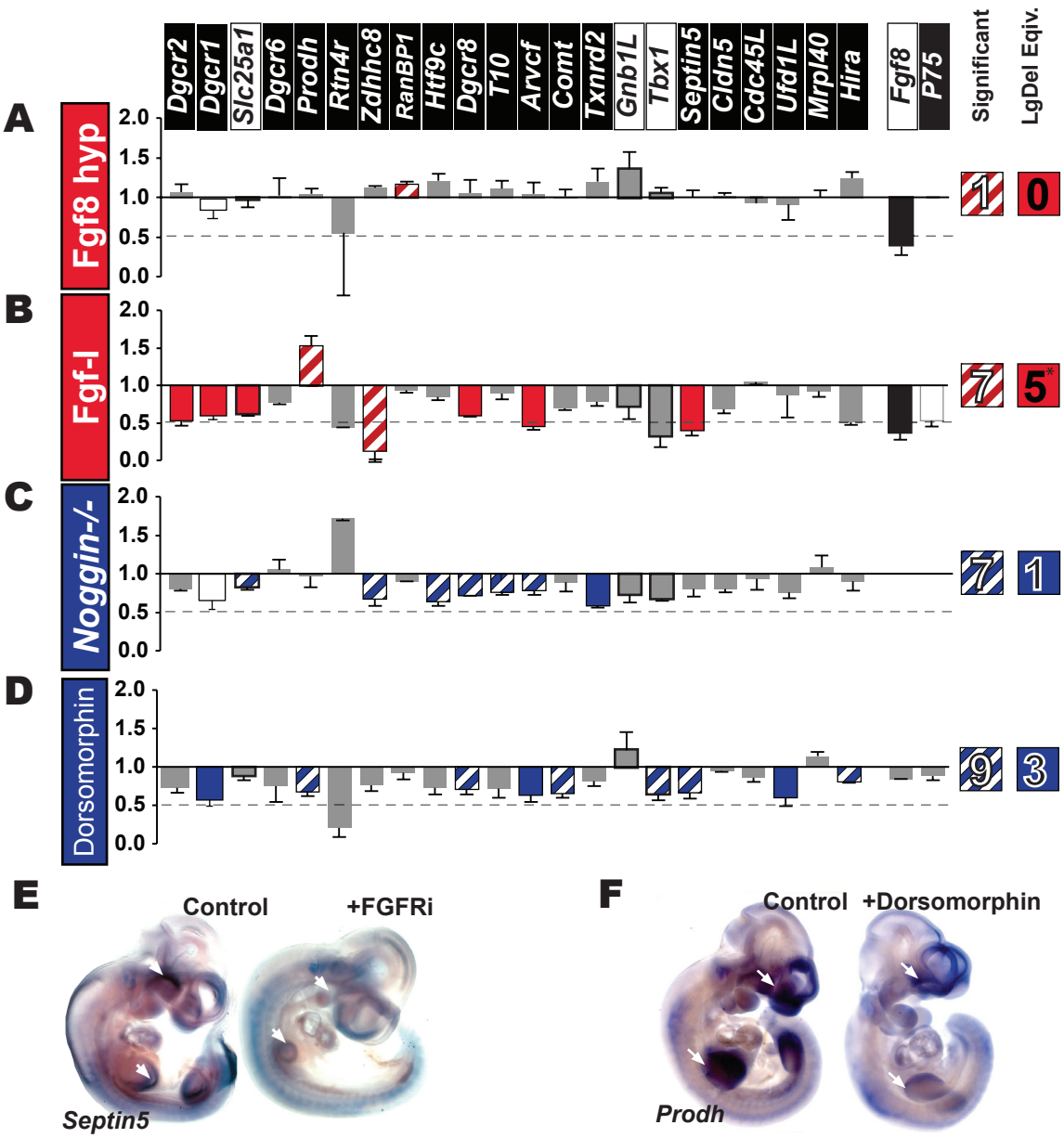
Figure 7: Altered RA signaling influences expression levels of a subset of 22q11 genes. Epithelium-restricted 22q11 genes are indicated by thicker black outlines on bars. (A) Expression of 16 genes is significantly decreased in highly dysmorphic E10.5 *Raldh2*$^{-/-}$ embryos (n=4; $p \leq 0.05$; t-test); 15 of these changes are statistically equivalent to that in *LgDel* embryos (solid purple bars; $p \geq 0.05$; 2-way ANOVA); however, significantly altered Fgf8 and P75 expression (solid black bars) indicates disrupted epithelial and mesenchymal integrity. (B) 24-hr sub-teratogenic RA exposure alters expression of 6 22q11 genes in E10.5 embryos (hatched purple bars; $p \leq 0.05$; n=4; t-test); however, none are statistically equivalent to that in *LgDel* embryos. (C) Transient inhibition of RA synthesis in E10.5 embryos using DEAB leads to diminished expression of six 22q11 genes to *LgDel* levels (solid purple bars). (D) Expression of the RA responsive gene, *Rarα,* is significantly increased following transient exposure of embryos to RA (50%) and DEAB (75%; $p \leq 0.05$, n=4 for each condition). (E) Expression of *Septin5* is increased in the frontonasal mass, branchial arches and limb bud (solid white arrows) in response to 24-hr RA treatment; *Dgcr8* expression in branchial arches, frontonasal mass and limb buds is decreased following DEAB mediated inhibition of RA synthesis (n=5 embryos/each probe).

Fgf and Bmp signaling and regulation of 22q11 gene expression

Fgf and Bmp signaling have less quantitatively detectable influence on maintaining normal levels of 22q11 gene expression at midgestation than either Shh or RA. In mildly dysmorphic *Fgf8*$^{neo/neo}$ embryos (approximately 40% of WT level of *Fgf8;* Figure 8A) a modest (approx.15%) but significant increase of *Ranbp1* is the only detectable 22q11 gene expression change (Figure 8A). PD173074 (128, 129), a small molecule inhibitor of FGF receptor-mediated signaling, disrupts Fgf signaling at M/E sites, confirmed by the down-regulation of *Mkp3* (130)). In PD173074-exposed embryos expression of seven 22q11 genes is significantly altered; five genes mirror *LgDel* changes, however, none parallel those in the *Fgf8*$^{neo/neo}$ embryos (Figure 8A,B). PD173074 also significantly diminishes *Fgf8*, with a similar trend for *P75* suggesting potential tissue loss at M/E sites. ISH in PD173074-exposed embryos shows altered 22q11 gene expression levels at M/E sites (Figure 8F); nevertheless, there is also hypoplasia at M/E sites suggesting diminished tissue volumes not seen following 22q11 deletion (131). Thus, PD173074-induced 22q11 gene expression changes may be secondary, due to tissue loss. Together, this data indicates that the contribution of Fgf signaling to normal 22q11 gene expression levels is less direct and substantial than that via Shh or RA.

Enhanced Bmp signaling due to loss of *Noggin* mediated Bmp antagonism (*Nog*$^{-/-}$(69)) significantly decreases one 22q11 gene (*Txnrd2*) by 50%, similar to *LgDel* ($p \leq 0.05$). Expression of 6 additional 22q11 genes is modestly (10-30%) but significantly diminished ($p \leq 0.05$) without detectable change of *Fgf8* or *P75* (Figure 8C). Inhibition of Bmp signaling by the small molecule antagonist

dorsomorphin (132) results in diminished expression of seven 22q11 genes, two of which are shared with *Nog*$^{-/-}$ embryos. Only three of the dorsomorphin-induce changes, however, are statistically similar to *LgDel* changes (Figure 8D), even though *Id1* a known Bmp target is enhanced by 30% (Figure 8G). ISH in dorsomorphin-treated embryos is consistent with altered expression levels of *Prodh*, the most substantially diminished 22q11 gene, without significant changes in pattern or morphology (Figure 8H). Thus, the contributions of Bmp signaling to maintenance of normal 22q11 gene expression levels, judged by numbers of genes whose expression is significantly altered by disrupted Bmp signaling, appear less substantial than those of Shh and RA.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Dgcr1
Slc25a1
Dgcr6
Prodh
Rtn4r
Zdhhc8
RanBP1
Htf9c
Dgcr8
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Arvcf
Comt
Txnrd2
Gnb1L
Tbx1
Septin5
Cldn5
Cdc45L
Ufd1L
Mrpl40
Hira
Fgf8
P75
Significant
LgDel Eqiv.
A
Fgf8 hyp
B
Fgf-I
C
Noggin-/-
D
Dorsomorphin
2.0
1.5
1.0
0.5
0.0
1
0
7
5*
7
1
9
3
E
Control
+FGFRi
Septin5
F
Control
+Dorsomorphin
Prodh

Figure 8: Fgf and Bmp signaling have limited impact on maintenance of normal levels of 22q11 gene expression. (A) *Fgf8*$^{neo/neo}$ embryos express 40% levels of wild-type *Fgf8* (solid black bar), but have no *LgDel*-like changes in expression of 22q11 genes; *Ranbp1* is modestly but significantly increased. (B) Transient inhibition of *FgfR* mediated signaling reduces *Fgf8* and *P75* expression as well as eight 22q11 genes; 6 gene expression changes are equivalent to *LgDel* (solid red bars) while Prodh and Zdhhc8 display increased and greatly reduced expression, respectively (hatched red bars). (C) *Noggin*$^{-/-}$ embryos have no measurable changes in epithelial or mesenchymal markers, but display significant and modest decrements in 7 genes that are not equivalent to *LgDel* embryo dosage (hatched blue bars), with one exception, *Txnrd2* (solid blue bar). (D) Transient pharmacological inhibition of Bmp signaling by dorsomorphin induces *LgDel*-like decrements in *Dgcr1*, *Arvcf*, *Tbx1* and *Ufd1l* (solid blue bars) as well as smaller magnitude changes in *Prodh*, *Dgcr8*, *Comt*, *Septin5* and *Hira* (hatched blue bars). (E) Inhibition of FgfR signaling results in 55% reduced expression of the Fgf target gene *Mkp3*. (F) Septin5 expression is severely diminished in the developing forebrain, branchial arches and limb buds; structures that are also severely morphologically compromised. (G) *Id1*, a Bmp target gene is upregulated following 24-hr dorsomorphin treatment. (H) *Prodh*, quantitatively down regulated by dorsomorphin, is diminished in the developing forebrain and limb buds.

Diminished dosage of 22q11 genes disrupts M/E signaling pathway gene expression

There may be reciprocal regulation of 22q11 genes and cardinal M/E inductive signals including Shh, RA, Fgfs, and Bmps. If this were the case, diminished 22q11 gene dosage should alter ligand expression, availability, abundance or activity of receptors and signaling co-factors. Accordingly, we first asked whether diminished 22q11 gene dosage modifies Shh, Fgf, RA or Bmp signaling pathways. To evaluate such interactions, we first analyzed mRNA levels for Shh, Fgf, or Bmp ligands, RA synthetic enzymes, which are essential for ligand production, and key receptors, signaling co-factors, and metabolic regulators for each pathway in whole E10.5 *LgDel* embryos (71).

Three of the four M/E signaling pathways appear altered—all due to decreased, rather than enhanced expression—by varying degrees as a result of diminished 22q11 gene dosage in *LgDel* embryos. Two Shh transcriptional mediators, *Gli1* and *Gli3,* decline in *LgDel* embryos (25%; $p \leq 0.05$; Figure 9A, far left). We detect no change in *Shh* ligands or receptors in whole embryo samples, including *Ptch1* and *Ptch2* which are unchanged despite known regulation by Shh-activated release of Gli transcriptional control (125, 133, 134). We next evaluated genes associated with RA synthesis, as well as RA receptors and co-factors. *Raldh3*, an RA synthetic enzyme highly enriched in the brain (135, 136) is diminished by nearly 50% ($p \leq 0.05$; Figure 9A, left center). Two receptors, *Rar*α and *Rar*β decrease by 28% and 19% respectively ($p \leq 0.05$). *Crabp1,* a binding protein thought to ensure ligand availability (137), declines by 30% ($p \leq 0.05$; n=4),

as does *Cyp26a1*, an RA degrading enzyme whose expression is RA-dependent (138) ($p \leq 0.05$, n=4). There were no changes in expression of two Fgf ligands, 2 receptors, 3 signaling regulators and an established Fgf-regulated gene (*Mkp3*;(130)) in the *LgDel* embryo (Figure 9A, right center). *Bmp* ligands are not significantly altered; however, *Id1* (a Bmp target gene) is modestly but significantly diminished (14%) as is the Bmp receptor *BmpR1* (17%) and two transcriptional cofactors, *Smad2* (16%), and *Smad4* (26%; $p \leq 0.05$, n=4; Figure 9A, right). Thus, based upon analysis of representative molecular mediators of the four signaling pathways, we do not detect changes in Fgf signaling molecules, Shh and Bmp mediators change modestly, and the most frequent, substantial and statistically significant expression changes for multiple elements (ligand-related, receptors, and co-factors) in mid-gestation *LgDel* embryos are seen for RA signaling.

$Tbx1^{+/-}$ and broader 22q11 deletion do not result in similar gene expression changes

Tbx1 has been proposed as a key, if not singularly explanatory, gene for several 22q11 phenotypes, especially those that reflect aberrant heart and brain development (71, 77, 139). If *Tbx1* alone is responsible for the majority of 22q11 phenotypes, and these phenotypes reflect altered Shh, RA, Bmp or Fgf signaling as suggested in several reports (93, 95, 96, 140), one would expect significant overlap between expression changes in Shh, RA or Bmp signaling molecules in *LgDel* and $Tbx1^{+/-}$ embryos. Of the 42 signaling intermediates we assessed, only 4 are significantly altered in $Tbx1^{+/-}$ embryos (Figure 9B): 2 Shh signaling mediators (*Smo* and *Sufu*), and 2 RA-associated genes (*Rar*α, *Raldh2*). Of these four, only

Rara overlaps with changes in E10.5 *LgDel* embryos; however, the change is of smaller magnitude. One Fgf signaling intermediate, *Spry4* (141) is altered in *Tbx1*$^{+/-}$ embryos, and no Bmp-related transcripts are changed (Figure 9B). Thus, there are fewer, and mostly divergent expression changes for M/E signaling pathway genes in *LgDel* versus *Tbx1*$^{+/-}$ embryos.

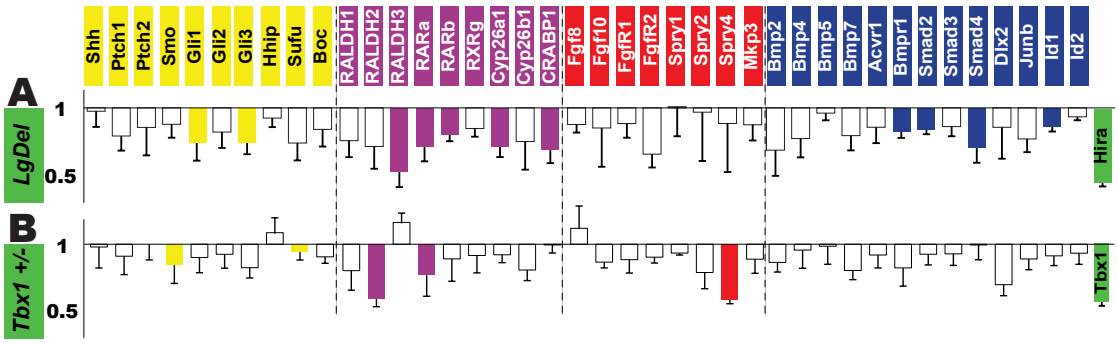
Shh
Ptch1
Ptch2
Smo
Gli1
Gli2
Gli3
Hhip
Sufu
Boc
RALDH1
RALDH2
RALDH3
RARa
RARb
RXRg
Cyp26a1
Cyp26b1
CRABP1
Fgf8
Fgf10
FgfR1
FgfR2
Spry1
Spry2
Spry4
Mkp3
Bmp2
Bmp4
Bmp5
Bmp7
Acvr1
Bmpr1
Smad2
Smad3
Smad4
Dlx2
Junb
Id1
Id2
A
LgDel
1
0.5
Hira
B
Tbx1 +/-
1
0.5
Tbx1

Figure 9: Divergent expression levels of M/E signaling genes—ligands or synthetic enzymes, receptors, transcriptional or metabolic cofactors—in *LgDel* and *Tbx1*$^{+/-}$ embryos. (A) Diminished expression of 2 Shh transcriptional co-factors (*Gli1* and *Gli3*), 5 RA synthetic enzymes, receptors or metabolic cofactors (*Raldh3*, *Rarα*, *Rarβ*, *Cyp6a1*, *Crabp1* respectively) and 4 Bmp receptors, transcriptional co-factors or targets (*Bmpr1*, *Smad2*, *Smad4* and *Id1*, respectively) in E10.5 *LgDel* embryos ($p \leq 0.05$; n=5; t-test). As a control, *Hira*, a 22q11 gene shows the expected 50% decrease. (B) Signaling gene expression in E10.5 *Tbx1*$^{+/-}$ embryos does not mirror *LgDel* changes. There is diminished expression of 2 Shh transcriptional co-factors (*Smo* and *Sufu*), 2 RA synthetic enzymes or receptors (*Raldh2* and *Rarα*, respectively), and 1 Fgf transcriptional co-factor (*Spry4*; $p \leq 0.05$; n=4; t-test). As expected, *Tbx1* expression is diminished by 50%.

Diminished 22q11 dosage results in altered Shh signaling at sites of M/E interaction

Our initial screens suggest reciprocal interactions between Shh signaling and 22q11 gene dosage may modulate M/E interactions and morphogenesis. To evaluate this hypothesis further, we measured the local "output" of M/E signaling via Shh at two clinically significant 22q11DS phenotypic sites, the heart and the forebrain, using either a "knock-in" (*Ptch2*:bGal;(142)) crossed into *LgDel* embryos to visualize and quantify local signaling independent of message levels, which may vary based upon stability and turnover. There are neither visible (Figure 10A) nor quantitatively detectable (Figure 10B) changes in Shh signaling in the head/forebrain of *LgDel* or *Tbx1*$^{+/-}$ embryos based on *Ptch2*:bgal transcription and activity. Shh signaling, however, apparently expands in the 1st and 2nd heart fields of *LgDel* embryos, and in parallel there is a 25% ($p \leq 0.05$, n=5) increase in soluble bgal activity (Figure 10A,B). Further analysis of local mRNA levels shows that *Shh* and its intermediates are unchanged in the head (Figure 10C, and data not shown); however, *Shh* is increased by approximately 50% in the heart, and mRNA levels for a co-receptor *Boc* decline modestly. Similar changes are not seen in *Tbx1*$^{+/-}$ embryos (Figure 10D,E). Local measurements of Shh-related mRNA levels support this conclusion (Figure 10F, and data not shown). Thus, based upon observation and measurement in *Ptch2* reporter embryos, and parallel assessment of local *Shh*-related gene expression, Shh signaling is altered in the heart of embryos carrying a deletion parallel to that in 22q11DS patients. There may be changes in Shh signaling in *Tbx1*$^{+/-}$ embryos; however, they do not include altered *Ptch2* activation or elevated *Shh* mRNA seen in *LgDel* embryos of similar age.

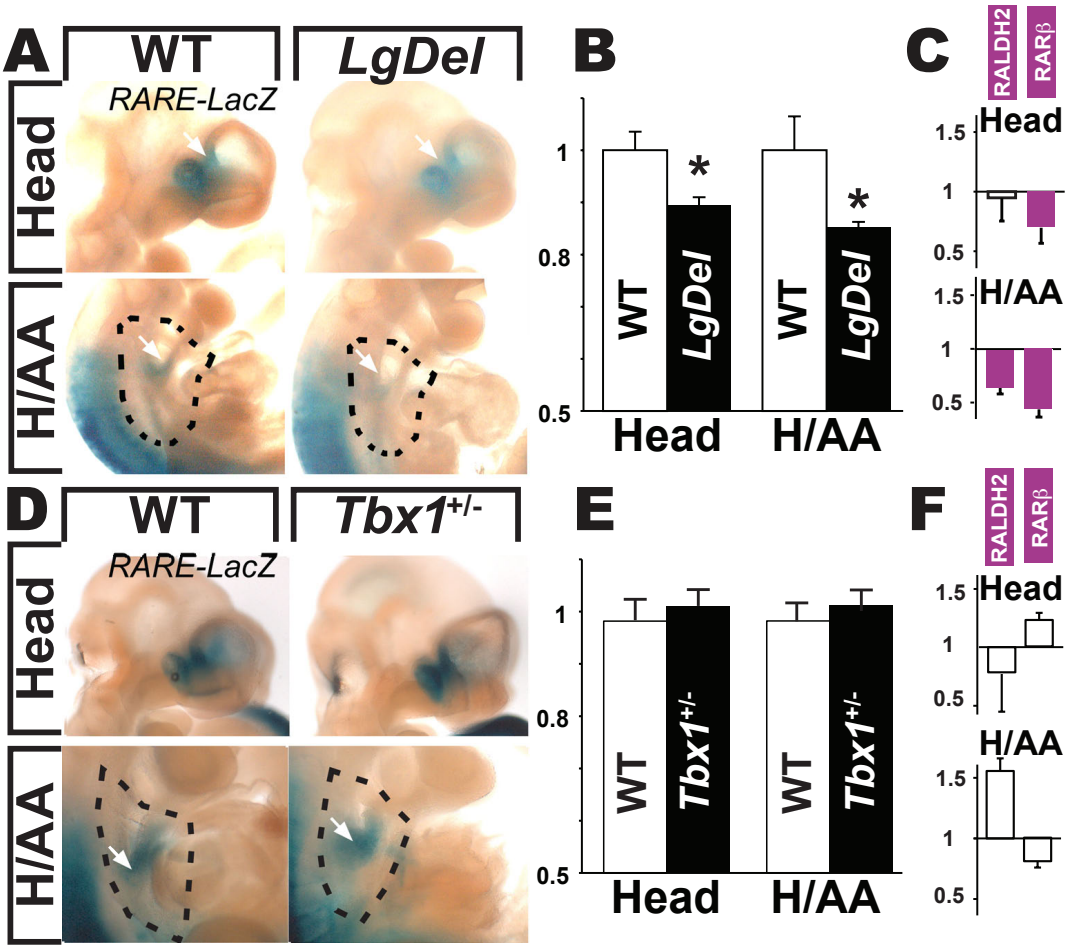
A
WT
LgDel
RARE-LacZ
Head
H/AA
B
1
0.8
0.5
WT
LgDel
WT
LgDel
*
*
Head
H/AA
C
RALDH2
RARβ
Head
1.5
1
0.5
H/AA
1.5
1
0.5
D
WT
Tbx1+/-
RARE-LacZ
Head
H/AA
E
1
0.8
0.5
WT
Tbx1+/-
WT
Tbx1+/-
Head
H/AA
F
RALDH2
RARβ
Head
1.5
1
0.5
H/AA
1.5
1
0.5

Figure 10: Shh signaling is increased in the developing heart in *LgDel*, but not *Tbx1*$^{+/-}$ E10.5 embryos. All embryos carry a β-galactosidase (β-gal) reporter under the control of the endogenous *Ptch2* promoter. (A) Increased β-gal labeling in pharyngeal endoderm of *LgDel* (H/AA, bottom right) versus wild type embryos (bottom left; 8 *LgDel* and 10 wild type embryos analyzed). There is no apparent change in the forebrain/head. (B) Increased β-gal activity in the heart and aortic arches (H/AA) of the *LgDel* embryos, without change in the forebrain/head. (C) Increased *Shh* and decreased *Boc* expression in the heart and aortic arches (H/AA), but not in the forebrain/head. (D) No visible difference in cranial or cardiac β-gal labeling in wild type and *Tbx1*$^{+/-}$ E10.5 embryos. (E) Equivalent β-gal activity in the head and heart (H/AA) of wild type and *Tbx1*$^{+/-}$ embryos. (F) There is no significant change in *Shh* or *Boc* expression in either head or heart (H/AA) of *Tbx1*$^{+/-}$ embryos.

Diminished 22q11 dosage results in altered RA signaling at sites of M/E interaction

To measure RA signaling activity we bred an RA-sensitive transgene in which βgal is expressed under the control of a DR5 retinoic acid response element (*DR5-RARE*; (57, 143, 144)) into the *LgDel* and *Tbx1* lines. In *LgDel* embryos, DR5-RARE-dependent RA signaling appears diminished, based upon intensity and extent of visible bgal labeling, in both the head/forebrain and heart (Figure 11A). In parallel, soluble bgal activity decreases modestly but significantly in the head/forebrain (12%; $p \leq 0.05$, n=6) and heart (15%; $p \leq 0.05$, n=6) of *LgDel* embryos (Figure 11B). Analysis of locally altered mRNA for RA receptors and signaling intermediates in microdissected E10.5 forebrain and heart is consistent with these changes. *Rarb* declines significantly in both the head/forebrain and heart of *LgDel* embryos (25-55%, Figure 11C; $p \leq 0.05$), as does the RA-synthetic enzyme, *Raldh2,* declines modestly (but not significantly) in the head, and more substantially in the heart (25% Figure 11C; $p \leq 0.05$). In contrast, in *Tbx1*$^{+/-}$ embryos, DR5-RARE expression, bgal activity and RA signaling mRNAs are not altered (Figure 11D-F). Apparently, RA signaling, at least that via DR5-RARE-mediated responses, is diminished in the heart and head/forebrain of *LgDel* embryos with diminished 22q11 gene expression, but not with heterozygous *Tbx1* deletion.

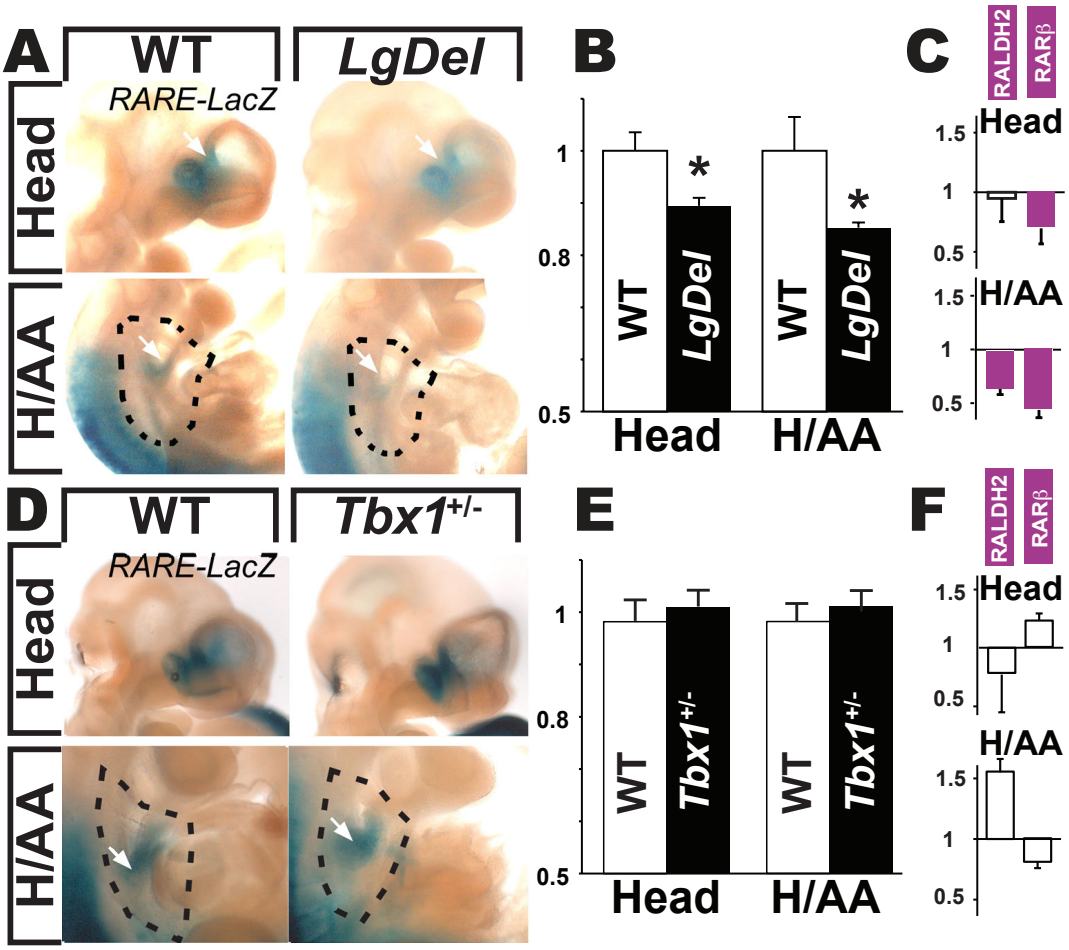
A
WT
LgDel
RARE-LacZ
Head
H/AA
B
1
0.8
0.5
*
*
WT
LgDel
WT
LgDel
Head
H/AA
C
RALDH2
RARβ
Head
1.5
1
0.5
H/AA
1.5
1
0.5
D
WT
Tbx1+/-
RARE-LacZ
Head
H/AA
E
1
0.8
0.5
WT
Tbx1+/-
WT
Tbx1+/-
Head
H/AA
F
RALDH2
RARβ
Head
1.5
1
0.5
H/AA
1.5
1
0.5

Figure 11: Diminished RA signaling in the head/brain and heart in *LgDel*, but not *Tbx1*$^{+/-}$ E10.5 embryos. All embryos analyzed express a β-gal reporter transgene expressed under the control of a RA responsive DR5-RARE. (A) Diminished β-gal staining in head (top right) and heart (bottom right) of E10.5 *LgDel* versus wild type embryos. (B) Diminished enzymatic activity in microdissected *LgDel* head and heart (H/AA) compared to wild type. (C) *LgDel* embryos have diminished expression of *Raldh2* and *Rarβ* in the heart (H/AA) but just *Rarβ* (solid purple bars) in the head ($p \leq 0.05$; n=6; t-test). (D) No differences in β-gal expression in the head or heart (H/AA) in *Tbx1*$^{+/-}$ versus wild type littermates. (E) β-gal activity is equivalent in *Tbx1*$^{+/-}$ and wild type littermates (n=4; t-test). (F) Expression levels of *Raldh2* and *Rarβ* in the head and heart (H/AA) are equivalent in *Tbx1*$^{+/-}$ and wild type littermates.

22q11 gene dosage and Shh signaling interact during morphogenesis

If 22q11 gene function critically influences Shh participation in mesenchymal/epithelial (M/E) signaling, one would expect additive dysmorphogenesis and altered cellular differentiation at 22q11DS phenotypic sites if Shh signaling is compromised in *LgDel*. Alternately, if claims of phenocopy are accurate (41), one would expect phenotypic equality between *LgDel* and *LgDel* embryos in which Shh signaling has been disrupted. We first addressed this issue genetically by crossing $Shh^{+/-}$ with *LgDel* mice. Although some anomalies were seen, we recorded no consistent dysmorphology in F1 littermates of the four possible genotypes (n=12 embryos; data not shown). To eliminate possible variation arising from alleles maintained on different genetic backgrounds (C57bl6:*LgDel* and S129:$Shh^{+/-}$), we briefly diminished Shh signaling in *LgDel* litters between E8.5 and E10.5 using low doses of cyclopamine, previously determined to be non-teratogenic at E10.5 (See Figure 6). qPCR analysis of 22q11 genes showed that, apart from *Zdhhc8*, cyclopamine-mediated disruption of Shh signaling does not elicit additional changes of 22q11 gene expression beyond the 50% diminished dosage in untreated *LgDel* embryos (compare Figures 5 and 12A). Nevertheless, the consequences of diminished Shh signaling reliably distinguish wild type and *LgDel* littermates morphologically (Figure 12B). The most affected *LgDel* embryos fail to develop any identifiable non-axial structures (limb buds, aortic or branchial arches). In contrast, wild type embryos—even those most compromised by cyclopamine—have appropriate structures for mid-gestation embryos. Due to the broad range of dysmorphology in *LgDel* embryos, we selected the least phenotypic wild type and *LgDel* embryos

for qPCR expression analysis to minimize confounding effects of tissue loss. While *Gli1* and *Gli3* are modestly decreased in untreated *LgDel* embryos (see Figure 9), cyclopamine treatment results in substantially diminished levels of all Shh-related signaling genes measured in both wild type and *LgDel* embryos. While some decrements were more pronounced in the *LgDel* (*Ptch1* - 79%, *Smo* - 69%, and *Gli1* - 61%; data not shown), the direction of change is uniform (Figure 12C). Thus, diminished 22q11 gene dosage sensitizes the embryo to deleterious consequences of disrupted Shh signaling; however, this sensitization does not reflect further decreased 22q11 gene expression.

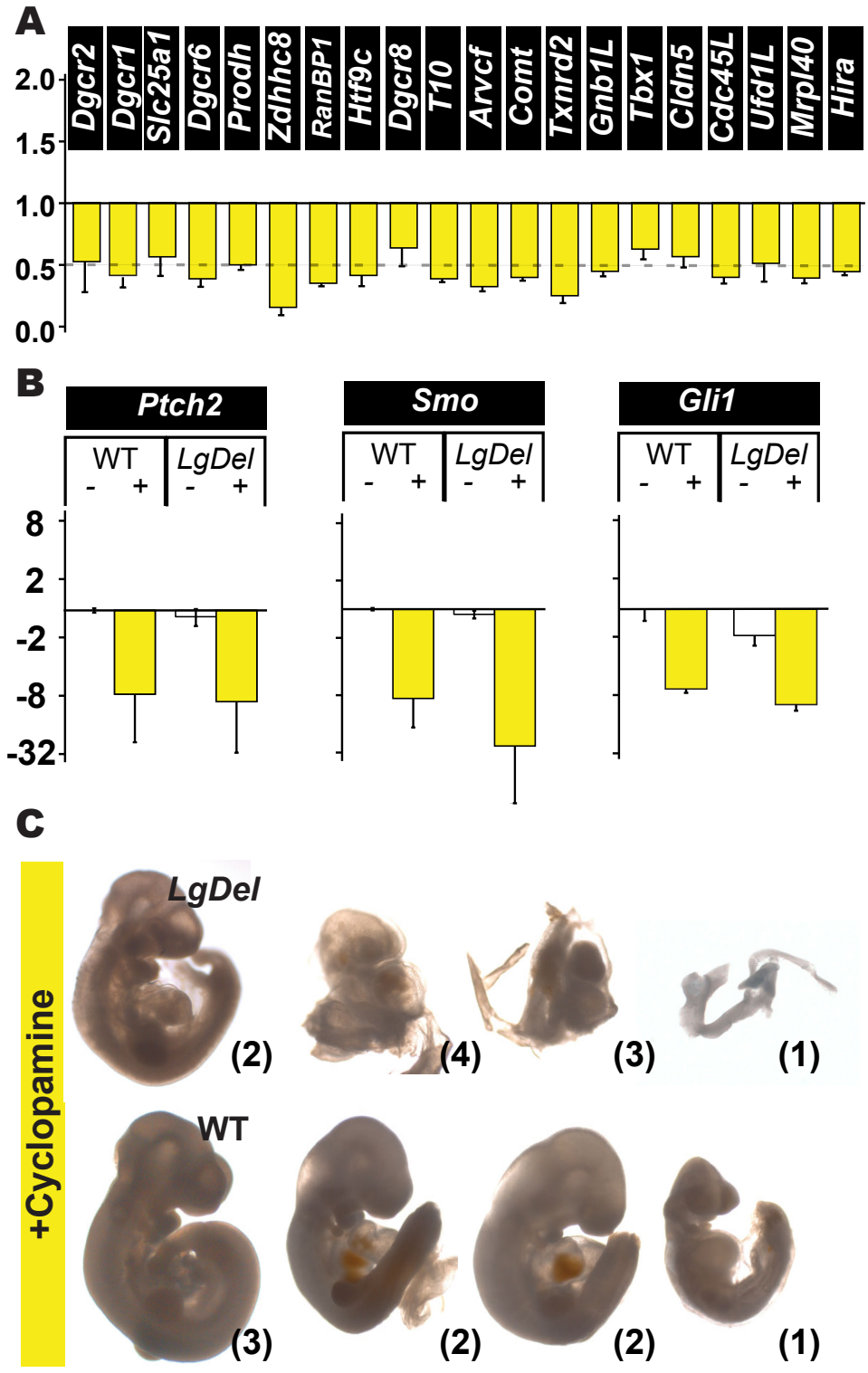
A
Dgcr2
Dgcr1
Slc25a1
Dgcr6
Prodh
Zdhhc8
RanBP1
Htf9c
Dgcr8
T10
Arvcf
Comt
Txnrd2
Gnb1L
Tbx1
Cldn5
Cdc45L
Ufd1L
Mrpl40
Hira
2.0
1.5
1.0
0.5
0.0
B
Ptch2
Smo
Gli1
WT
LgDel
- + - +
8
2
-2
-8
-32
C
+Cyclopamine
LgDel
(2)
(4)
(3)
(1)
WT
(3)
(2)
(2)
(1)

Figure 12: Diminished 22q11 gene dosage sensitizes embryos to altered Shh signaling. (A) qPCR shows no significant effect of 48 hour cyclopamine treatment on 22q11 gene expression in the E10.5 *LgDel* embryo. mRNA levels were measured in embryos with relatively milder phenotypes (see below). (B) *Ptch2*, *Smo* and *Gli1* expression is similarly altered in *LgDel* and wild type littermates, indicating no significant effect of 22q11del on expression of these Shh signaling co-factors (2-way ANOVA). (C) *LgDel* embryos (top half) are more morphogenetically sensitive than wild type littermates (bottom half) to inhibition of Shh signaling by cyclopamine. Each embryo shown represents an apparent degree of phenotypic change (mildest, left; greatest, right), and numbers in parentheses indicate how many embryos were considered to be in each class, based on scoring by 2 independent observers, of 10 *LgDel* and 8 wild type embryos.

22q11 gene dosage and RA signaling interact during morphogenesis

If the proposed "phenocopy" (40) between 22q11DS and RA loss-of-function reflects a linear relationship where loss of 22q11 gene dosage results in aberrantly decreased RA signaling, then increasing RA signaling in *LgDel* embryos might rescue 22q11DS phenotypes. Thus, we asked whether modest elevation of RA via maternal circulation, (see Figure 7), mitigates phenotypic and gene expression differences between mid-gestation wild type and *LgDel* embryos. A transient increase of RA between E8.5 and E10.5 in *LgDel* embyros does not alter expression levels of mesenchymal or epithelial 22q11 genes beyond 50% (Figure 13A); however, it does substantially disrupt cranial and cardiovascular morphogenesis of *LgDel* embryos compared to wildtype littermates (Figure 13B). *LgDel* embryos treated with RA exhibit significant defects in mesencephalic neural tube closure as well as 3rd and 4th pharyngeal arch artery morphogenesis (Figure 13B and 15B). *LgDel* embryos also display aberrant transcriptional responses at RA-signaling-related loci. Expression levels of *Raldh1* and *Raldh2,* RA synthetic enzymes primarily expressed in the mesenchyme (136), are significantly diminished in the *LgDel* embryo (6- and 4-fold, respectively; $p \leq 0.01$) in response to increased RA availability (Figure 13C). This response is not seen in treated wild type or untreated *LgDel* embryos. *Rarα* and *Rarβ* are upregulated as expected in wild type embryos (6.8-fold and 2-fold, respectively) in response to increased RA (126); however, these genes are divergently regulated in RA-treated *LgDel* embryos (Figure 13C). Not only is RA unable to elevate *Rarα* in the *LgDel*—it diminishes *Rarβ,* a critical receptor for mesenchymal RA activity (145, 146)—a reversal of the wild-type response. A

two-way ANOVA, comparing expression of RA-responsive loci in wildtype, RA-treated, 22q11 deleted or *LgDel* embryos treated with RA, confirms statistically significant interaction between the genetic deletion and acute pharmacological treatment with all-trans RA ($p \leq 0.001$). Deletion of the DiGeorge critical region significantly affects tissue-response to variability in RA signaling, interfering with M/E signaling mediated morphogenesis of structures affected in DiGeorge syndrome between E8 and E10.5.

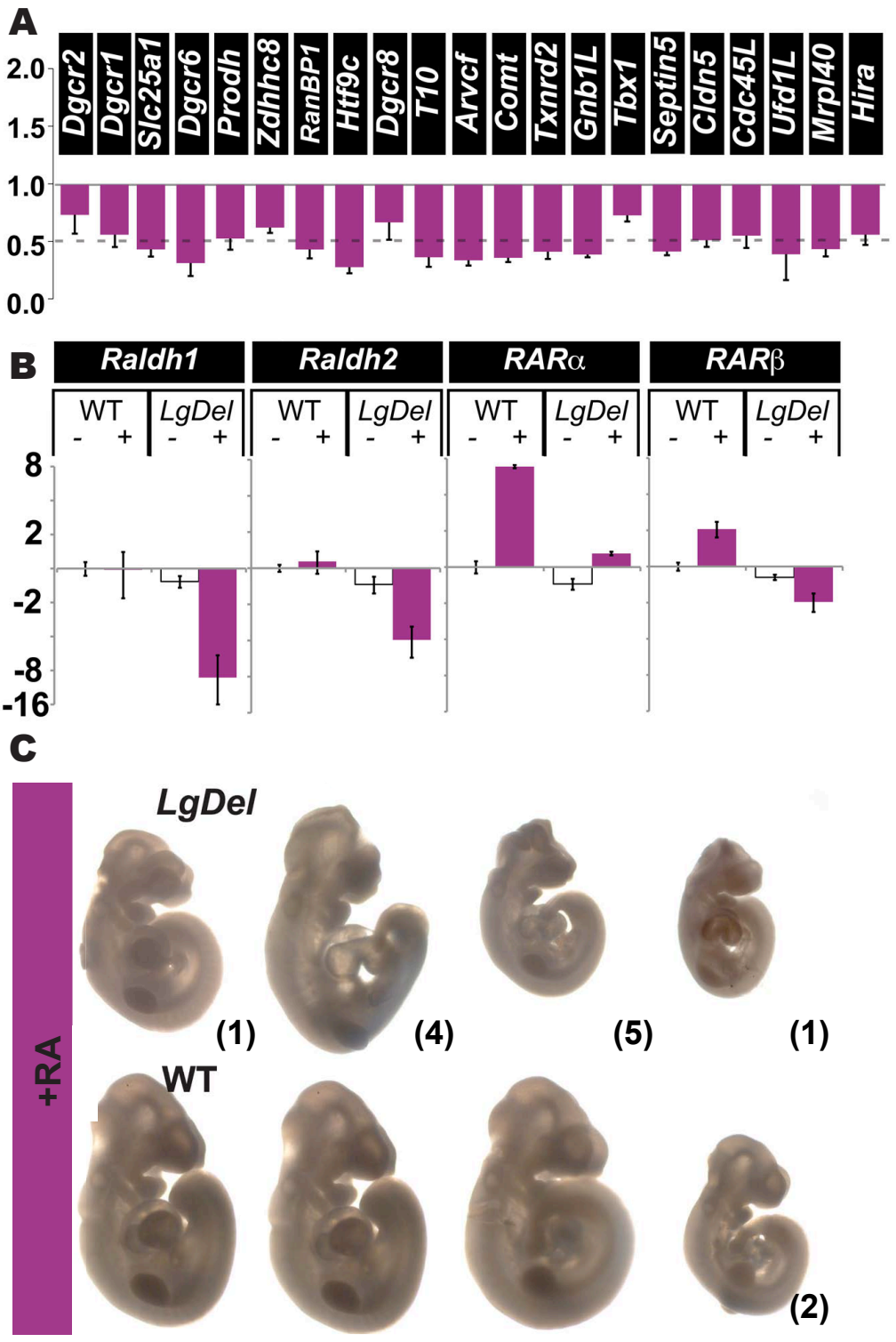
A
Dgcr2
Dgcr1
Slc25a1
Dgcr6
Prodh
Zdhhc8
RanBP1
Htf9c
Dgcr8
T10
Arvcf
Comt
Txnrd2
Gnb1L
Tbx1
Septin5
Cldn5
Cdc45L
Ufd1L
Mrpl40
Hira
2.0
1.5
1.0
0.5
0.0
B
Raldh1
Raldh2
RARα
RARβ
WT
LgDel
- + - +
8
2
-2
-8
-16
C
LgDel
+RA
WT
(1)
(4)
(5)
(1)
(2)

Figure 13: Diminished dosage of 22q11 genes sensitizes embryos to sub-teratogenic RA exposure. (A) Increased RA availability between E8 and E10 does not have a significant effect on 22q11 gene expression in E10.5 *LgDel* embryos. (B) Distinct expression changes in RA signaling-related genes in *LgDel* embryos: *Raldh1* and *Raldh2* are significantly diminished in *LgDel* but not wild type littermate embryos in response to RA ($p \leq 0.05$; n=3; 2-way ANOVA). *Rar*α and *Rar*β are increased in wild type, but not *LgDel* embryos ($p \leq 0.05$; n=3; 2-way ANOVA). (C) RA-exposed *LgDel* (top half) and wild type littermates (bottom half) have varying degrees of morphogenetic change in a sample of 15 *LgDel* and 19 wild type littermate embryos from 4 litters scored by 2 independent observers. Representative phenotypic changes are shown for RA exposed *LgDel* embryos, and numbers in parentheses indicate frequency of each class We only noted mild dysmorphology (smaller size) in 2 wild type embryos

22q11del sensitizes cranial and cardiovascular morphogenesis to RA availability

If specific aspects of morphogenesis that are compromised at sites of M/E signaling in 22q11DS depend upon interactions that are sensitive to both 22q11 gene dosage (115) and RA signaling levels (96, 126, 147), then modifying RA signaling in the *LgDel* embryo should result in specific additive or new phenotypes. Thus, we determined whether elevated RA results in consistent, distinct, intensified phenotypes in the brain or heart, sites of clinically significant developmental anomalies in 22q11DS (10, 148). A transient and sub-teratogenic increase in RA availability has a significantly different effect on *LgDel* embryos versus similarly treated wild type or *Tbx1*$^{+/-}$ embryos. 11 out of 15 (73%) *LgDel* embryos exposed to RA showed neural tube closure deficits at E10.5 (Figure 13B, Table 2). Exencephalic defects are seen in the posterior neural tube (1/15; 7%), rhombencephalon (4/15; 26%) and most frequently in the mesencephalon (6/15; 40%; Figure 14A). Neither wild type nor *Tbx1*$^{+/-}$ littermates had any gross cranial phenotypes in response to increased RA availability. To evaluate potential cell biological consequences that accompany this gross morphological change, we assessed patterning, proliferative capacity and cytological integrity of wild type as well as RA-treated wild-type and *LgDel* forebrain precursors. We assessed localization of Nkx2.1 (ventral forebrain marker; Figure 14B) and Pax6 (dorsal forebrain marker), distribution and frequency of phospho-Histone3 (PH3, M-phase precursors; Figure 14B), Nestin (radial glial/neural stem cells; Figure 14C), b-catenin (apical polarity; Figure 14D) and Tuj1 (early neurons; Figure 14E). Transiently increasing RA availability does not appear to disrupt dorso-ventral Nkx2.1/Pax6 patterning nor does it have a significant effect on proliferation of

forebrain progenitors based upon frequency of PH3 labeled cells. In addition, there were no detectable, *LgDel*-related disruptions of radial glial morphology, polarity or early neuronal differentiation in the nascent marginal zone. Thus, it is likely that the effect of altered RA availability on neural tube closure in the *LgDel* is due to either a proliferative change not measured by PH3 or a defect in progenitor cell patterning and identity that does not alter Pax6, Nkx2.1, Nestin, b-catenin and Tuj1.

We also analyzed 4th pharyngeal arch artery hypoplasia, a major clinical issue in a large subset of 22q11DS patients that is recapitulated with varying penetrance in several 22q11DS mouse models including *LgDel* mice, is (71, 73). If RA signaling interacts with 22q11 genes to direct pharyngeal arch artery (PAA) morphogenesis, then altering RA-signaling in the *LgDel* should further modify PAA development. As reported previously, 62% of untreated *LgDel* embryos display 4th pharyngeal arch defects (5/8, Table 2). There were no observable effects in the hearts of wild type embryos treated with RA (0/11, Figure 15A, Table 2). In contrast, increased RA availability in *LgDel* embryos increases the penetrance and severity of 4th arch defects to 100% (8/8, Figure 15B, Table 2), and intensifies *LgDel* 4th arch hypoplasia to complete lack of 4th arch development (5/8, Table 2). To assess if additive phenotypes reflect diminished dosage of *Tbx1* alone or additional 22q11 genes, we analyzed RA-treated *Tbx1*$^{+/-}$ embryos. 4/10 (40%) untreated *Tbx1*$^{+/-}$ embryos had hypoplastic 4th PAA, consistent with previous reports (Figure 15C). Only 4/7 (57%) *Tbx1*$^{+/-}$ embryos treated with RA show 4th arch defects versus 100% of the RA/*LgDel* cohort (Table 2). In addition, while the most affected *LgDel* embryos have a highly dysplastic 3rd PAA and lack 4th and 6th PAA, the most affected *Tbx1*$^{+/-}$ embryos have mildly dysplastic 3rd

PAA, lack 4th PAA but retain the 6th PAA (compare right panel of 10B and 10C). Deletion of the entire 22q11 critical region, including, but not of *Tbx1* alone, clearly compromises cranial and cardiac morphogenesis by sensitizing the affected tissues to variation in RA signaling.

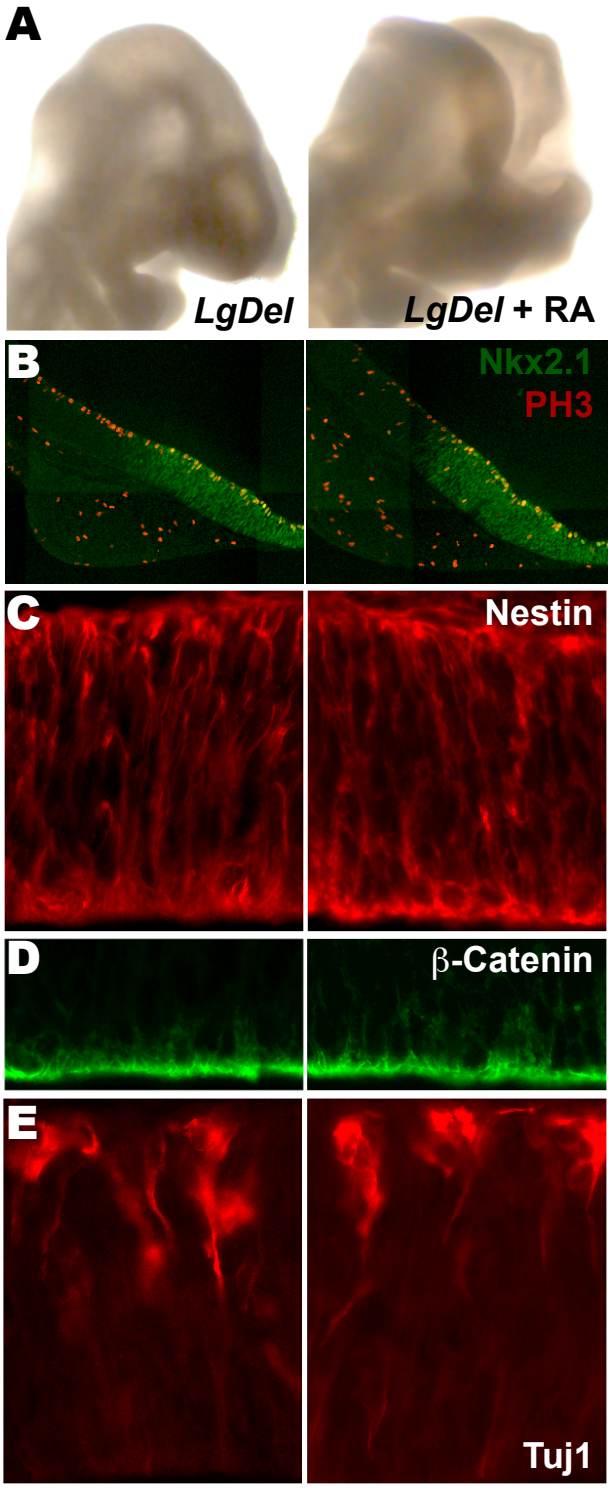
A
LgDel
LgDel + RA
B
Nkx2.1
PH3
C
Nestin
D
β-Catenin
E
Tuj1

Figure 14: Diminished dosage of 22q11 genes results in a higher frequency of exencephaly, without detectable changes in cellular organization of the forebrain neuroepithelium. (A) RA treatment has no noticeable effect on neural tube closure in E10.5 wild type embryos (left) but results in exencephaly in *LgDel* embryos (right; 73%, see Table1). (B) Nkx2.1 pattern and PH3 frequency (no statistical difference, data not shown) are not altered in the *LgDel* ventral forebrain following RA exposure. (C) Nestin staining of forebrain radial glia reveals no obvious difference in these presumed neural stem cells in the early forebrain. (D) Polarity of forebrain precursors (evaluated by b-catenin labeling) and (E) initial neuronal differentiation (Tuj1 labeling) are not noticeably disrupted by RA exposure.

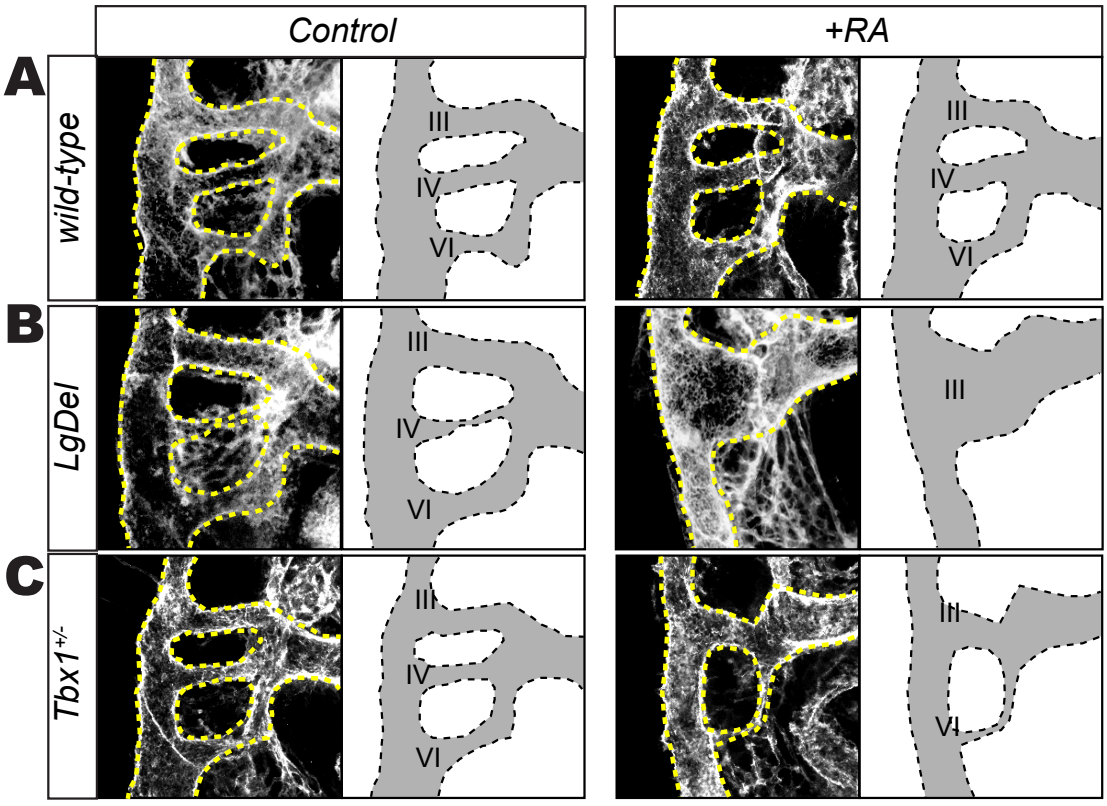
Control
+RA
A
wild-type
B
LgDel
C
Tbx1+/-
III
IV
VI

Figure 15: RA exposure results in enhanced pharyngeal arch artery (PAA) dysmorphology in *LgDel* embryos. Confocal microscopic images of the 3rd, 4th and 6th PAAs of CD-31 (PECAM) antibody stained untreated (left panels) and RA treated (right panels) E10.5 wild type (A), *LgDel* (B) and *Tbx1*$^{+/-}$ (C) embryos. Yellow dotted lines indicate the outline of each PAA on the confocal Z-stack. Outline of PAA pattern, size and shape is shown to the right of the image. RA exposure has no significant effect on wild type embryos (compare left and right panels of A). Untreated *LgDel* embryos have the expected 4th PAA hypoplasia, while those treated with RA are severely dysmorphic (panel B, far right), exhibiting dysplastic 3rd PAA and absence of 4th and 6th PAAs. *Tbx1*$^{+/-}$ embryos treated with RA maintain development of the 3rd and 6th PAA but display dysplasia of the 4th PAA (far right, panel C).

Tables

Table 1

	Neural Tube defect classification						
				Most anterior region of exencephaly			
	Total Scored	Normal	Exencephalic	Spinal Cord	Rhombencephalon	Mesencephalon	Telencephalon
LgDel+RA	15	4 (26%)	11 (73%)	1 (7%)	4 (26%)	6 (40%)	0
Tbx1$^{+/-}$+RA	10	10 (100%)	0	0	0	0	0

	4rth Pharyngeal Arch phenotype			
	Total	Normal	Hypoplastic	Absent
WT	**7**	7	0	0
WT+RA	**5**	3	2	0
LgDel	**8**	3	5	0
LgDel+RA	**8**	0	3	5
Tbx1$^{+/-}$	**10**	6	4	0
Tbx1$^{+/-}$+RA	**7**	3	2	2

Table 2

	4rth Pharyngeal Arch phenotype			
	Total	Normal	Hypoplastic	Absent
WT	**7**	7	0	0
WT+RA	**5**	3	2	0
LgDel	**8**	3	5	0
LgDel+RA	**8**	0	3	5
Tbx1$^{+/-}$	**10**	6	4	0
Tbx1$^{+/-}$+RA	**7**	3	2	2

CHAPTER 4

DISCUSSION

Reciprocal interaction between 22q11 genes and cardinal inductive signals—especially Shh and RA—is crucial for normal gene expression, activity, and morphogenesis at sites of M/E interaction and phenotypic change in 22q11DS. Shh maintains normal expression levels of all epithelial and several mesenchymal 22q11 genes at these sites, while full dosage of mesenchymal 22q11 genes establishes an appropriate context for production and activity of RA at sites of M/E interaction, especially the brain and heart. Diminished dosage of 22q11 genes sensitizes the embryo to variations in Shh and RA signaling that would be aphenotypic in wild type embryos. Apparently, loss of 22q11 gene dosage constricts the range of Shh and RA signaling that is morphogenetically acceptable. Thus, by disrupting Shh and RA signaling, 22q11 deletion may lead to increased severity of brain and cardiovascular anomalies (149) due to decreased embryonic tolerance to signaling variability, potentially similar to naturally occurring signaling variation encountered during gestation (150).

Shh maintains 22q11 gene expression

An appropriate level of epithelial Shh signaling is needed to maintain normal expression of all epithelial and a significant number of mesenchymal 22q11 genes. Constitutive or acute loss of Shh in *Shh*$^{-/-}$ and cyclopamine-treated

embryos, respectively, results in *LgDel*-like decrements of *Slc25a1, Gnb1L* and *Tbx1* in the epithelium and *Dgcr6*, *Ht9c, Arvcf, Septin5* and *Hira* in the mesenchyme; all are implicated in 22q11DS phenotypes (71, 121). In contrast, complete loss of *Gli3* has no significant effect on levels of 22q11 genes, indicating that Shh-mediated maintenance of 22q11 gene expression levels is independent of Gli3. Homozygous loss of *Hira* (121) and *Tbx1* (73) result in significant dysmorphology in the vasculature, heart or brain—paralleling, to some extent, phenotypes in $Shh^{-/-}$ (41). Several Shh-dependent processes, including skeletal development and vasculogenesis, are disrupted in 22q11DS. Thus, apparent similarities between 22q11DS and Shh loss of function phenotypes (40, 41) may be due to disrupted Gli3-independent Shh signaling that diminishes 22q11 gene expression to levels seen following 22q11 deletion.

Decreased 22q11 gene dosage likely disrupts morphogenesis by altering Shh signaling levels. Expression of both Gli transcriptional effectors (*Gli1*) and activator/repressors (*Gli3*) are diminished in *LgDel* embryos. Nevertheless, Shh signaling output, as well as Shh expression, is increased in the heart based on our reporter as well as qPCR analysis in microdissected embryonic PAA/heart tissue. In the heart, it is possible that epithelial Shh, by maintaining 22q11 gene expression in the mesenchyme, sets up a feedback mechanism that further regulates epithelial Shh signaling centers (Figure 16A, (151)). Diminished dosage of Shh responsive 22q11 genes may disrupt this relationship leading to aberrant Shh expression, including the local increase in Shh expression we found in the heart (Figure 16B). Such local, potentially compensatory mechanisms may either serve to mitigate loss of 22q11 gene activity or further modulate M/E interactions required for appropriate morphogenesis. Recent studies have shown

that Shh is required for cardiac neural crest survival (46) and maintenance of heart field progenitor proliferation (47). Thus, the local gain in Shh activity we report here may defray some cardiovascular consequences of decreased gene dosage of Shh-regulated 22q11 genes like *Hira* and *Tbx1* (92, 121, 152), while unchanged Shh signaling may actually place other phenotypic sites at greater morphogenetic risk (Figure 16B).

RA signaling pathways are disrupted by 22q11 deletion

Our data suggests that appropriate 22q11 gene dosage is required to maintain the integrity of retinoid signaling centers, most of which are located in the mesenchyme at 22q11DS phenotypic sites (138). Decreased expression in *LgDel* embryos of two RA-regulated receptors (*Rar*α and *Rar*β(58, 153);) and a signaling cofactor (*Crabp1*), all known to be expressed primarily in mesenchyme (154), may interrupt RA production or activity at its mesenchymal sources (152, 155, 156) and disrupt RA signaling and metabolism in target tissues. Indeed, our indicator embryo assay shows that *LgDel* target tissues have significantly less DR5-RARE-dependent transcription, an event often seen in epithelia adjacent to mesenchymal RA sources. Conversely, diminished 22q11 dosage may elicit ectopic RA-activity in the *LgDel* by decreasing expression of a metabolic P450 enzyme that catabolizes RA in non-target tissues (*Cyp26a1* (157);). Thus, deletion of multiple 22q11 genes critical to RA production or signaling in the mesenchyme may interfere with the ability of tissues to generate, transmit or metabolize RA resulting in locally decreased RA signaling output in the developing brain and heart (Figure 16B).

Appropriate levels of RA signaling are critical for patterning in the forebrain as well as pharyngeal arches and heart (152) (158, 159). Thus, the decreased RA signaling we identified in the head and heart of *LgDel* embryos may contribute to CNS and vascular defects in 22q11DS by disrupting numbers or distribution of RA-activated precursors (FIX REFS). Our data indicates that heterozygous deletion of multiple 22q11 genes but not *Tbx1* alone alters RA signaling. If *Tbx1* was solely responsible for 22q11 phenotypes at sites of M/E induction—many of which are thought to reflect altered RA signaling (71, 95, 139)—one might expect significant or complete overlap between $Tbx1^{+/-}$ and *LgDel* embryos. With the exception of the epithelially expressed *Rarα* (145), there is no overlap of RA signaling gene expression changes in $Tbx1^{+/-}$ and *LgDel* mid-gestation embryos. *Tbx1* deletion may result in RA signaling changes restricted to epithelial tissues while broader 22q11 deletion compromises both mesenchymal and epithelial compartments. Accordingly, deletion of the entire 22q11 region likely disrupts mesenchymal ability to generate, transmit and metabolize RA, leading to altered proliferation and differentiation in targets of local mesenchymal RA sources in the brain (160) and outflow tract (158, 161).

Diminished 22q11 dosage sensitizes the embryo to altered signaling

Our results indicate that full 22q11 gene dosage establishes a dynamic range of Shh and RA signaling within which morphogenesis proceeds normally (Figure 16A,B). Embryos with 50% dosage of 22q11 genes transiently challenged with cyclopamine or RA have significantly enhanced developmental anomalies not seen in their wild type littermates. CNS mid-line development, antero-

posterior and dorso-ventral patterning as well as pharyngeal arch artery morphogenesis are critically dependent upon appropriate levels of Shh and RA signaling (55, 159, 162). Previous observations of dose-dependent increase in phenotypic severity in response to genetic (**REF**) or pharmacological manipulations of Shh and RA in wild type embryos suggest that modest variation may be tolerated; however, large changes disrupt development (124, 148). In the face of signaling volatility, we have seen greater morphogenetic disruption in *LgDel* embryos than wild-type littermates. Thus, full 22q11 gene dosage may mediate adaptation to normal variation in Shh or RA signaling due to diet (163), drug or alcohol abuse (148) or toxic exposure (164, 165). Similarly, polymorphisms of Shh and RA-related genes in 22q11-deleted embryos might move signaling levels outside of optimal limits (Figure 16B), modifying the severity or penetrance of brain and cardiovascular phenotypes. Loss of 22q11 gene dosage may preferentially alter the ability of the mesenchyme, where 22q11 genes are selectively expressed, to buffer against signaling changes. Such changes can disrupt developmental capacity in both mesenchyme and epithelium leading to developmental aberrations in DiGeorge syndrome.

The dynamic range of Shh signaling necessary for midline and non-axial development may be severely contracted by 22q11 deletion (Figure 16B; solid yellow). Total loss of Shh, critical for dorsoventral patterning and cell specification, results in severe midline deficits and heart developmental anomalies (48). Exposing *LgDel* litters to low levels of the Shh inhibitor cyclopamine during a critical stage for neural tube closure and dorsoventral patterning results in complete collapse of midline as well as non-axial development restricted to *LgDel* embryos. Thus, deletion of 22q11 genes leads to

a contraction of the low end of the range of Shh signaling within which morphogenesis proceeds normally (Figure 16B). Additionally, increased Shh signaling output in the heart fields of *LgDel* embryos implies diminished 22q11 gene dosage likely drives signaling to the low end of the dynamic range leading to a compensatory increase to maintain morphogenesis. This may contribute to incomplete penetrance of heart phenotypes in *LgDel* embryos (71). Exposing these embryos to cyclopamine in an attempt to rescue morphological deficits likely pushes them further outside the 'dynamic range' that can support normal development. Therefore, full 22q11 gene dosage buffers Shh signaling variability due to genetic or environmental causes by establishing a 'dynamic range' to preserve normal morphogenesis.

Morphogenetic adaptability within a dynamic range of RA signaling also depends on 22q11 gene dosage. Aberrant, reversed transcriptional responses in *LgDel* embryos indicate that deletion of several upstream 22q11 genes disrupt downstream synthesis or transmission of RA. In the face of these RA signaling changes, *LgDel* embryos are unable to maintain the appropriate signaling range necessary for normal development. Abnormal expression decrements of synthetic enzymes (*Raldh1* and *Raldh2*) and ineffective expression of receptor loci (*Rara* and *Rarb*) in the face of increased RA availability likely pushes the embryo past a threshold of morphogenetically acceptable RA signaling (Figure 16B; dotted line). Increased RA availability in RA-teratogenized and *Cyp26a1* mutants (where RA variation is not efficiently modulated (59)) results in spina bifida, exencephaly and pericardial ballooning (59, 162). Parallel deficits in rhombencephalic neural tube closure and 3rd, 4th and 6th pharyngeal arch arteries in *LgDel* embryos in response to increased RA indicates that diminished dosage

of 22q11 genes, particularly in RA-producing and responsive mesenchyme, likely constricts RA dynamic range, disrupting RA-mediated brain and pharyngeal morphogenesis in the face of signaling volatility. Thus, our data indicates that multigenic 22q11 gene loss, not just *Tbx1* alone, constricts the dynamic range of RA signaling within which embryogenesis can proceed normally. The loss of adaptability to RA signaling variation results in significant defects in neural tube closure and pharyngeal arch artery morphogenesis.

A dynamic range for Shh and RA signaling and phenotypic variation in 22q11DS

One of the most defining characteristics of 22q11DS patients with either the minimal critical deletion or the larger typical deletion (149, 166), is the highly variable severity and penetrance of phenotypes (10). Comparisons of 22q11DS phenotypes to RA and Shh-related anomalies in humans and mouse-models suggest parallel or serial convergence of these pathways and morphogenesis of these structures (40, 41). It is likely that genetic polymorphisms or environmental insults that modify Shh or RA signaling may concatenate with the altered dynamic developmental range that we have described in the *LgDel* resulting in cell differentiation deficits in the forebrain as well patterning deficits in the second heart field. Several recent studies suggest that modifier loci associated with Shh and RA signaling may interact with DiGeorge genes (93, 95, 118). We have shown for the first time that likely causes for this variation are altered Shh and RA signaling due to diminished dosage 22q11 genes beyond *Tbx1,* and resulting divergent morphogenetic interactions at sites of M/E induction. Our data also indicates that a dynamic range of optimal Shh and RA-signaling may

be constricted in 22q11DS patients leading to phenotypic variation in response to changes in signaling that would normally not cause dysmorphology. Accordingly, allelic variation associated with Shh or RA signaling as well as early fetal exposure to Shh or RA agonists or antagonists such as oxysterols (167), ethanol (148) and glyphosate-based herbicides (168) in deleted individuals may modify heart, limb, face, or brain development. Thus, phenotypic variability in 22q11DS may reflect the inability of M/E interactions to buffer stress of sudden or chronic changes in Shh and RA signaling engendered by additional genetic lesions or environmental stimuli.

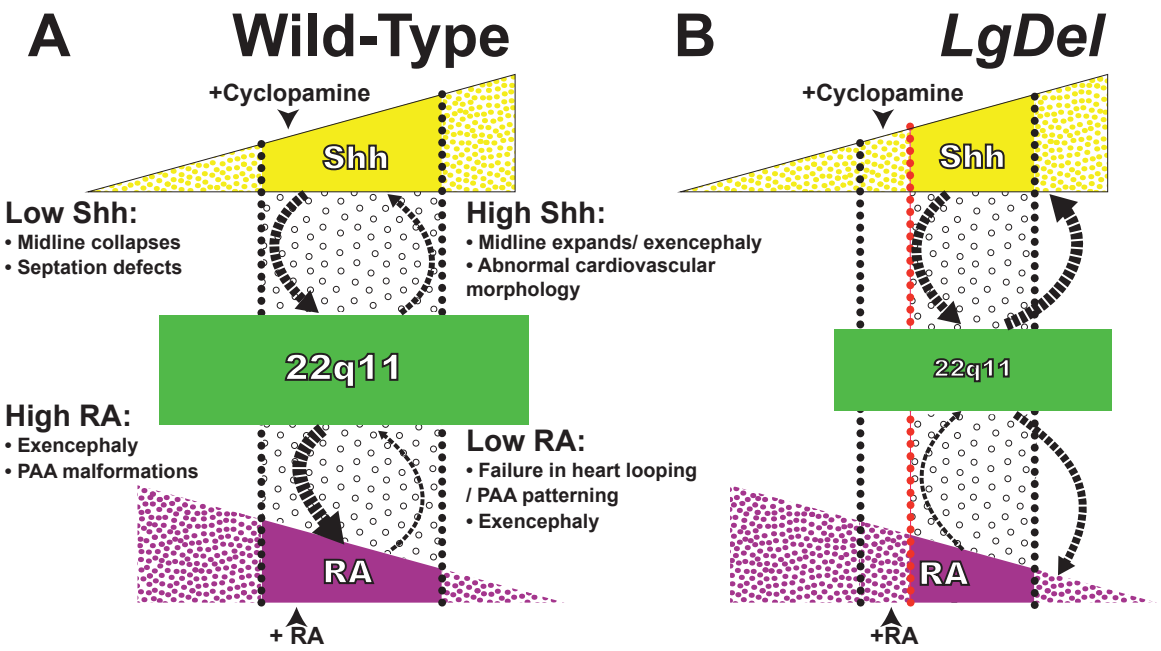
A
Wild-Type
B
LgDel
+Cyclopamine
Shh
Low Shh:
• Midline collapses
• Septation defects
High Shh:
• Midline expands/ exencephaly
• Abnormal cardiovascular morphology
22q11
High RA:
• Exencephaly
• PAA malformations
Low RA:
• Failure in heart looping / PAA patterning
• Exencephaly
RA
+ RA
+Cyclopamine
Shh
22q11
RA
+RA

Figure 16: Full 22q11 gene dosage supports, and diminished dosage constricts, a dynamic range of Shh and RA signaling. (A) Range of Shh (solid yellow) and RA (solid purple) signaling within which embryonic development proceeds normally in wild type embryos. Extreme signaling deviations (dotted yellow and purple) leads to disrupted morphogenesis (either side of dotted black lines). Shh maintains 22q11 gene expression (thick arrow), and full 22q11 gene dosage influences levels of Shh signaling (thin arrow). Normal dosage of 22q11 genes is required to maintain morphologenetically appropriate RA signaling (thick arrow) and RA availability modestly influences 22q11 expression (thin arrow). Wild type embryos develop normally since variable levels are "buffered" within an acceptable dynamic range (solid blocks) via 22q11 genes, even when challenged by cyclopamine (+Cyclopamine) and RA exposure (+RA). (B) *LgDel* embryos have contracted dynamic range within which morphogenesis proceeds normally (new boundary indicated by red dotted line; compare to black dotted line in panel A). Diminished 22q11 gene expression (smaller green box) increases Shh signaling (thick arrow-top right) and diminishes RA signaling (thinner arrow, lower right), particularly at sites of M/E interaction that are phenotypically compromised in 22q11DS. Disrupting Shh and RA signaling in *LgDel* embryos using identical sub-teratogenic dosages of cyclopamine and RA causes enhanced dysmorphogenesis in *LgDel* embryos because signaling levels fall outside of the constricted dynamic range.

MATERIAL AND METHODS

Mice

The University of North Carolina at Chapel Hill (UNC-CH) Division of Laboratory Animal Medicine maintained colonies of wild type Institute of Cancer Research (ICR), *Shh*$^{+/-}$, *Gli3*$^{+/-}$, *Fgf8*$^{neo/+}$, *Raldh2*$^{+/-}$, *Nog*$^{+/-}$, *Tbx1*$^{+/-}$, RA (*DR5-RARE-LacZ*;(57)) and Shh-sensitive (*Ptch2-LacZ*, Deltagen) transgenic mice. All experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committee at The University of North Carolina at Chapel Hill or the George Washington University School of Medicine. Mice carrying a heterozygous deletion in chr.16 from Idd to Hira were maintained on a C57BL/6J background (*LgDel* mice; (71)). In this study, the deletion was inherited paternally. Timed-pregnant litters (day of vaginal plug = E0.5) were generated by mating wild type mice, or mice heterozygous for each mutation, independently. Timed-pregnant females were sacrificed by rapid cervical dislocation and embryos were dissected and collected for RNA isolation, or fixed and appropriately processed for *in situ* hybridization, whole mount antibody or *β-galactosidase* substrate staining.

Quantitative PCR (qPCR)

E10.5 embryos were harvested, dissected and homogenized in TRIzol (Invitrogen). Total RNA was isolated per manufacturers instructions, and cDNA

synthesized as previously described (131). Power Sybr green reagent (Applied Biosystems), with 200 μM forward and reverse primer, was used to amplify and quantify specific transcripts from whole embryo or dissected regional cDNA samples using the ABI 7500 system (Applied Biosystems).

Immunohistochemistry and *In Situ Hybridization*

For immunohistochemistry and in situ hybridization, embryos were fixed overnight in 4% paraformaldehyde (PFA) in PBS at 4°C. Immunohistochemistry was performed on 20um cryostat sections as previously described (115). Immunofluorescently labeled sections were imaged using a Nikon epifluorescense (Eclipse 80i) microscope in combination with Surveyor© system (Objective Imaging). For whole mount immunofluorescent staining, embryos were serially dehydrated into methanol, stored overnight at -80°C, and freeze-thawed five times. Following rehydration into PBS, specimens were incubated overnight at 4°C with gentle rocking in a blocking buffer comprised of 5% DMSO, 0.2% Triton X-100, and 5% normal goat serum, in PBS. Following incubation with primary antibodies (Rat anti-CD31; BD-Pharmingen) in blocking buffer overnight at 4°C, specimens were rinsed 5-6 times over an 8 hour period and subsequently labeled with Alexa-Fluor 488 or 546 conjugated anti-rat secondary antibodies overnight in blocking buffer at 4°C. Following further rinsing and dehydration into MeOH, samples were rendered optically transparent with a 2:1 solution of benzyl alcohol:benzyl benzoate (BABB) and imaged while in BABB in a custom chamber on a Zeiss LSM 510 confocal microscope. Z-Stacks were collected and 2D projections were created utilizing the Zeiss LSM image processing software. Primary antibodies were commercially

obtained [CD-31 (BD Pharmingen), PH3 (Cell Signaling Technology), Nestin (Millipore), Tuj1 (Covance), p-Smad (Cell Signaling Technology), b-catenin (Sigma), and Nkx2.1 (Santa Cruz)]. *In situ* hybridization was performed on whole-mount embryos as described previously (131) and these hybridized embryos were imaged on a Leica M420 macroscope.

***β-galactosidase* staining and enzymatic activity quantification**

Embryos were harvested and dissected in ice cold PBS and either fixed in 0.1% glutaraldyhyde or lysed in 2X ONPG (*ortho*-Nitrophenyl-*β*-galactoside) lysis buffer (Promega ONPG assay system) for whole mount staining or a soluble *β*-galactosidase activity assay, respectively. Whole mount fixed embryos were stained for *β*-galactosidase activity with X-gal substrate (Promega) as previously described (152). Head or heart tissue was dissected and solubilized in 2X lysis buffer (promega ONPG assay system) and total activity of *β*-galactosidase in the sample was detected as a function of enzymatic hydrolysis of ONPG into *ortho*-nitrophenol. Samples were spectrophotometrically analyzed at 420nm on a ELX808 ultra microplate reader (Bio-Tek Instruments).

Pharmacological treatment of embryos

Pregnant wild-type dams were injected with 10mg/kg RA (Sigma), 100mg/kg DEAB (Sigma), 80mg/kg cyclopamine (LC Laboratories), 50 mg/kg PD173074 (Sigma) or 10 mg/kg dorsomorphin (Sigma) at E9.5 and embryos were harvested 24-hours later. Pregnant *LgDel* dams were injected with RA (10-20mg/kg) or cyclopamine (80mg/kg) twice (E8.5 and E9.5) prior to sacrifice on E10.5.

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Conflict of Interest Statement

There have been no influences that might bias the work reported or in the Results, or modify any interpretations, implications, or opinions stated in the manuscript.

CONCLUSION

Copy number variations are the most common type of structural variations in the human genome (169). There is increasing evidence of the role of CNVs in human disease. 22q11 deletion syndrome (22q11DS) is one of the most common "copy number variant" (CNV) genetic disorders currently known (1:2000) (4). The major phenotypes associated with 22q11DS, including cardiovascular and cognitive and behavioral deficits, indicates that early development of the brain and pharyngeal apparatus is affected (4, 9-11) due to diminished dosage of 22q11 genes. It is likely that the deletion specifically disrupts second heart field directed morphogenesis in the heart and compromises early patterning and differentiation of the telencephalon, leading to disrupted cortical circuitry in the brain (115, 170, 171). We examined the role of diminished dosage of 22q11 genes in M/E signaling mediated phenotypes. Our data supports the role for full dosage of 22q11 genes in maintaining a tissue microenvironment that is able to sustain normal morphogenesis even in the face of variable Shh and RA signaling. Deletion of the region and loss of 22q11 gene function in the cell cycle machinery, mitochondrial metabolism and microRNA processing likely interferes directly with RA signaling while disrupting Shh mediated morphogenesis in a parallel manner.

The Heart:

Neural crest provides the mesenchyme for the 3rd, 4rth and 6th pharyngeal arches. The pharyngeal mesenchyme contributes first the myocardium and then the smooth muscle that forms the arterial pole (170). This region of the splanchnic mesoderm that forms the arterial pole is called the secondary heart field. As some of the secondary heart field progenitors differentiate, others continue to proliferate to generate enough cells to provide both myocardium and smooth muscle to the arterial pole (172). These proliferative cells are located more caudally in the field and are adjacent to the pharyngeal endoderm, which produces SHH (47). RA is synthesized and active in the anterior portion of this field. Thus it is likely that while Shh maintains proliferation, RA (in conjunction with other signals) provides differentiation signals to these progenitors.

My data shows that 22q11 genes are mainly expressed in the mesenchyme of the pharyngeal pouches and brain, (except 3 that are restricted to the epithelium) and that heterozygous deletion results in 50% expression of all the genes. Diminished dosage of 22q11 genes also results in defects of the myocardium and the outflow tract, possibly due to altered proliferation or differentiation of second heart field cardiac precursors. Thus it is likely that diminished dosage of a subset of 22q11 genes (Ranbp1 (173), Cdc45l (174), Hira (175), Ufd1l (176) and Septin5 (177)), thought to modulate cell cycle may disrupt proliferation of the caudal second heart field progenitors resulting in a reduced contribution to the developing outflow tract. My data shows that Shh activity is necessary to maintain expression of all these cell-cycle genes except for Cdc45L. Another gene implicated in cell cycle regulation, Htf9c, is also dependent on Shh for expression. Thus, it is likely that the increased Shh transcript as well as

activity in the heart fields is a compensatory mechanism in response to loss of 22q11 genes. Thus it is likely that diminished expression of these 22q11 genes associated with proliferation as well as several metabolic genes whose activity might support cell division result in decreased proliferation in the caudal second heart field and a compensatory increase in Shh signaling is necessitated to mitigate this effect. Supporting this hypothesis is my observation that decreasing Shh signaling in *LgDel* by administering cyclopamine between E8.5 and E10.5 completely derails morphogenesis of the embryo. Thus it is likely that several cell cycle, structural and metabolic genes contained within the 22q11 region are involved in proliferation of caudal second heart field progenitors alongside SHH and the increase in Shh in *LgDel* is likely a compensatory mechanism to mitigate the drop in proliferation. It is important to investigate the cellular and molecular mechanisms that govern this interaction to better understand cardiovascular disease.

Once these second heart field cells have been generated in the caudal aspect of the secondary heart field they must migrate up toward the outflow structure and then differentiate into myocardial and smooth muscle structures. RA is a differentiation signal produced in the mesenchyme in the anterior part of the second heart field. In conjunction with TGFb signaling (including Bmp) it drives the specification, differentiation, septation and morphogenesis of the outflow tract (101, 158). My data shows that there is decreased RA signaling in the developing heart and aortic arch of *LgDel* mid-gestation embryos. It is likely that multiple 22q11 genes are involved in the transmission and response to the RA ligand. Increased RA availability disrupts pharyngeal arch artery patterning and alters outflow vessel morphogenesis and development. In addition, our data

shows that the response of the tissue to RA is significantly disrupted in the *LgDel*. I show that embryos with diminished dosage of 22q11 genes have a markedly different transcriptional response of RA-responsive loci when RA signaling is altered. My data also shows that RA may also regulate expression of a small subset of 22q11 genes (*Dgcr6*, *Ranbp1*, *Dgcr8* and *Septin5*). *Dgcr6* has putative functions as cell surface receptors and its loss results in cardiovascular defects. Interesting RA is required to inhibit expression of *Ranbp1*, a cell cycle related gene. It is likely that by inhibiting *Ranbp1* (a gene positively regulated by Shh), RA could be shifting the proliferative nature of the progenitor cells to a more myocardial fate. *Dgcr8* is part of a complex that processes microRNA and may have an important role in establishing cell identity. *Septin5* being a structural protein could potentially be involved in changes in cell shape and structure and thus could have a role in cell differentiation driven by RA. Thus, it is likely that the microenvironment of the anterior secondary heart field where RA is actively involved in outflow tract differentiation is critically altered by diminished expression of a large set of 22q11 genes involved in establishing cell structure, cell cycle, cell metabolism and microRNA processing as a part of the RA signaling network.

Thus, our data indicates that, in the caudal secondary heart field, Shh-dependent expression of a subset of 22q11 genes likely direct the proliferation of the progenitors that will eventually migrate into the anterior aspect of the secondary heart field where interactions between RA, BMP and several 22q11 genes will drive the differentiation of these cells leading to outflow tract septation and morphogenesis. Future work should focus on an integrative approach to delineating the cellular and molecular roles of gene dosage within

these morphogenetic networks and the implications of gene dosage on establishing a genetic buffer for signaling variability.

Brain:

The cortex develops from a morphologically uniform ventricular zone located in the dorsocaudal part of the telencephalic vesicles. The development of the forebrain is governed by critical M/E interactions between signaling from the surface ectoderm, genes and signals expressed in the mesenchyme (mainly neural crest derived) and the developing neuroepithelium of the telencephalic vesicles. Regional identity and morphogenesis in the cortex is directed in part by Shh, Fgf, Bmp, Wnt and RA signaling centers producing secreted molecules. These centers are initially located along the edges and midline of the neural plate and later along and flanking the midline of the telencephalic vesicles (84, 178).

Work in our lab suggests diminished dosage of a substantial set of 22q11 genes expressed at sites of mesenchymal/epithelial interaction in the brain likely compromises early morphogenesis (122). Subsequently, diminished dosage in the *LgDel* mouse results in deficits in proliferation of the basal cortical progenitors and migration of newly-born interneurons into the cortex. These deficits lead to changes in the post natal brain such as decreased progeny of basal progenitor cells populating layer 2-4 of the cortex and altered laminar distribution of mature interneurons. We suggest that these defects could result from diminished dosage of a subset of 22q11 genes important for proliferation and migration (115). It is also likely that decreased expression of a subset of mitochondrial 22q11 genes involved in energy metabolism might compromise postnatal synaptogenesis. It is possible that modification of 22q11-dosage dependent development during initial forebrain patterning, later neurogenesis/ migration or post-natal

synaptogenesis may account for the variable behavioral pathology seen in 22q11DS.

While Shh, and to a lesser extent RA, maintain 22q11 gene expression, my analysis of early telecephalic stage embryos indicates that diminished dosage of 22q11 genes results in decreased RA signaling in the brain, but does not have any significant effect on Shh signaling. Altering RA availability in the *LgDel* leads to lack of neural tube closure and exencephaly, reminiscent of RA teratogenesis. RA, synthesized in the neural crest derived mesenchyme, is a potent inducer of neuronal identity (156) and is required for early forebrain patterning and morphogenesis (179). Recent reports show that meningeal RA, which signals to the, is critical for the formation of the cortrex (56). Thus, diminished expression of cell-cycle and metabolic 22q11 genes, who likely play a role either in RA signal transduction and response or RA synthesis, likely alter the ability of lateral mesodermal tissue in the developing forebrain to synthesize/transmit RA, mispatterning forebrain progenitors. Additionally, diminished RA signaling in 22q11DS likely disrupts the neurogenic gradient of RA necessary for appropriate corticogenesis later in development (56).

Our analyses of E10.5 *LgDel* embryos that have been treated with RA do not show any overt changes in cellular identity, polarity or proliferation in the forebrain. It is likely that changes in early signaling might result in forebrain and cortical deficits later in development. Thus, the interaction of 22q11 genes with RA signaling should be studied on a molecular, cellular and anatomic level once the cortical plate has emerged.

It is likely that the constricted range of signaling that can support appropriate morphogenesis in 22q11DS patients likely explains the variability of

phenotypes seen in this disorder. Future studies need to investigate how the multigenic interaction between 22q11 genes and Shh and RA signaling networks, in the developing brain as well as secondary heart fields established this dynamic range. In particular, the inability to sustain normal development in the face of signaling volatility needs to be elucidated, as it will provide insights into phenotype variability in 22q11DS as well as other congenital anomalies that display variation in incidence and severity. Thus, my data supports the need for embryological screening of 22q11DS and concurrent evaluation of prenatal drug; diet ands supplementation in light of apparent increased developmental sensitivity in CNV compromised embryos.

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